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COMPARISON OF PHENOTYPIC CONFIRMATION TEST (PCT) AND DOUBLE DISC SYNERGY TEST(DDST) FOR DETECTION OF EXTENDED SPECTRUM β-LACTAMASES(ESBL) PRODUCERS AMONG GRAM - NEGATIVE BACILLI IN A TERITARY CARE HOSPITAL

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ABSTRACT **Introduction and Aim:** The use of β-Lactam antibiotics is challenging due to the emergence of various antimicrobial resistance (AMR) mechanisms particularly by extended-spectrum β-lactamases (ESBLs) producing bacteria. They are increasing in number due to their mutations. The aim of the study was to compare the sensitivity between Phenotypic confirmatory test and Double disc synergy test of ESBLs producing Gram negative bacilli. **Material and Method:**672 pus samples were processed from January to May 2022, by standard procedure of identification and antimicrobial susceptibility testing. Isolated Gram- negative bacilli screened for ESBLs production. They confirmed by Phenotypic confirmatory test and also subjected for double disc synergy test for comparison of sensitivity of two tests. **Results:** A total of 554(82.4%) bacterial isolates were obtained from 672 pus samples. Out of 554 isolates,72% gram-negative bacilli and 10%-gram positive bacteria. In Gram -negative bacilli, 40.3% ESBL producers. The most frequent Klebsiella 17.2% followed by Escherichia coli 13%, Proteus 4% Pseudomonas 5% and Citrobacter 2%. Among the ESBL producers, Phenotypic confirmation test detected 88.7% and Double-disc synergy test detected 74.4%, are significant. **Conclusion:** Early detection of ESBL producers will help the clinician for appropriate treatment, our study showed that phenotypic confirmatory test is more sensitive than Double disc synergy test for detection of ESBL producing Gram-negative bacilli.

KEYWORDS : Extended Spectrum of β-Lactamases, β-lactamases, Phenotypic confirmatory test, Double disc synergy test.

INTRODUCTION:

The increasing resistance of microorganisms to antimicrobials is the greatest risk to human health. β-Lactam antibiotics are used for treating most of the nosocomial and community-acquired infections that are caused by Gram-negative bacteria mainly due to *Enterobacteriaceae* family. However, the use of β-Lactam antibiotics is challenging due to the emergence of various antimicrobial resistance (AMR) mechanisms particularly by extended-spectrum β-lactamases (ESBLs)[1].

EXTENDED SPECTRUM β LACTAMASES(ESBLs):

ESBL-are a group of plasmid-mediated, diverse, complex and rapidly evolving enzymes [2]. They are capable of hydrolysing penicillin's, broad-spectrum cephalosporins, and monobactams, but they do not affect the cephamycin's and carbapenems, and their activity is inhibited by clavulanic acid. In addition, ESBL-producing organisms are frequently exhibiting resistance to other antimicrobial classes due to associated resistance mechanisms, which may be either chromosomally or plasmid-encoded. The widespread use of third-generation cephalosporins was believed to be the major cause of mutations in these enzymes that leads to the emergence of plasmid-encoded ESBLs. These ESBLs were transferred between bacteria by plasmids, which were in turn spread by clonal distribution between hospitals and countries through patient mobility [3]. Carbapenems, are often used to treat infections caused by ESBL-producing *E. coli and Klebsiella species. However*, carbapenems enzymes recognize almost all hydrolysable β-lactams, and most are resistant to inhibition by all commercially viable β-lactamase inhibitors[4].

The Ambler molecular classification and the Bush-Jacoby-Medeiros functional classification are the two most commonly used classification systems for β-lactamases [5]. ESBLs are classified under Bush's functional class 2be.These enzymes are plasmid -mediated and are derived from point mutation of TEM on SHV β-lactamases. The ESBLs derived from TEM-1, TEM-2, or SHV-1 differ from their progenitors by as few as one amino acid. This results in a profound change in the enzymatic activity of the ESBLs, so that they can now hydrolyze the third-generation cephalosporins or aztreonam [5,6]. ESBLs are most commonly found in the Enterobacteriaceae family like Klebsiella species, Escherichia Coli and also seen in other members of Enterobacteriaceae are Salmonella, Proteus and Citrobacter species. They also reported in NON-fermenters like Pseudomonas aeruginosa, Acinetobacter species.

Hence the **Aim** of the present study is to detect ESBL production in Gram-negative bacilli in pus samples and their confirmation by Phenotypic confirmation test (PCT) and is compared with Double disc synergy test (DDST) to indicate the use of simple, reliable and cost - effective test method.

MATERIALS AND METHODS:

- **Study design**: Prospective study
- **Study period**: June to November 2022
- **Specimens included**: Pus samples
- **Sample size**: 672 pus samples of patients coming to microbiological laboratory.
- **Inclusion criteria**: Pus samples from patients admitted in tertiary care hospital.
- **Exclusion criteria:** Patients who were on previous antibiotic treatment and of Paediatric age group.

Culture and Antimicrobial susceptibility testing:

These samples were collected from various wards of Government General Hospital, Kakinada. All these samples were processed by Gram's Staining and inoculated on Blood agar and MacConkey agar and incubated at 37°C aerobically overnight. All the isolated were identified by their colony morphology, staining characters, pigment production, motility and other relevant biochemical tests as per standard methods of identification tests (Koneman7th edition). All the Gram-negative bacilli were subjected for Antibiotic susceptibility testing by disc diffusion technique as per standard CLSI guidelines [11].

Antimicrobial susceptibility testing:

All Gram-negative bacilli were tested for antimicrobial susceptibility on Muller Hinton agar with 0.5 McFarland standard inoculums using kirby-Bauer disk diffusion method. The Choice of antibiotic disks was selected as per CLSI guidelines [11]. The Antimicrobial Discs Were Amoxicillin/Clavulanic acid(20/10µg) Ampicillin(10µg), Amikacin(30µg), Cotrimoxazole(30µg), Gentamicin(30µg), Ciprofloxacin(5µg), Cefotaxime(30µg), Ceftriaxone(30µg), Ceftazidime(30µg), Ceftazidime/Clavulanic acid(30µg), Imipenem(30µg), Meropenem(30µg), Gentamicin(10µg), Ciprofloxacin(30µg), Piperacillin and Tazobactam PIT (30µg), were used. Zones Of Inhibition Was Recorded as Sensitive or Resistant According to CLSI Guidelines[11].

Methods for ESBL detection:

Phenotypic screening for ESBL Production:

The ESBL screening test was performed by the standard disk diffusion method by using ceftazidime(30µg),Ceftriaxone(30µg) and Cefotaxime(30µg) .More than one antibiotic disc were used for screening to improve the sensitivity of ESBLs detection as recommended by CLSI guidelines .These 3 antibiotic discs were placed at a gap of 20mm and overnight incubated at 37^{0}C .The isolates with reduced susceptibility to cefotaxime, ceftazidime and ceftriaxone around the disks were suspected as ESBLs producers.

Antibiotic disc	Zone diameter
Cefotaxime (CTX)-30µg	<27mm
Ceftazidime (CAZ)- 30µg	<22mm
Ceftriaxone (CTR)- 30µg	<25mm

All the suspected ESBL producers were subjected for

Phenotypic confirmatory test (PCT):
Confirmation of the ESBL producing isolates was done by the phenotypic confirmatory test according to CLSI recommendation. In this test, third generation cephalosporin i.e., ceftazidime (30 µg) disc alone and in combination with clavulanic acid (10 µg) were used. Ceftazidime disc were placed 20 mm distance from one side and ceftazidime combined with clavulanic acid (30/10 µg) were placed on other side of the inoculated plate. After overnight incubation at 37 0C, diameter of zone of inhibition was measured. The organism is said to be ESBL producer, when the zone of inhibition around the ceftazidime disc is ≥5mm compared to zone of inhibition around ceftazidime disc alone.

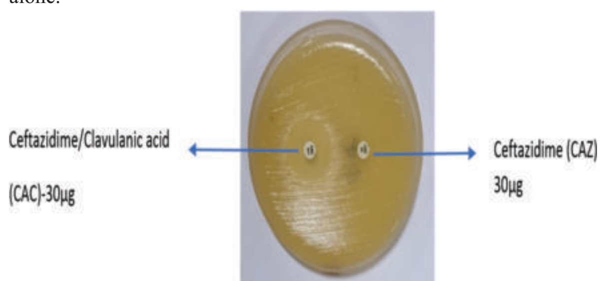

FIGURE-1
PCDDT showing >5mm increase in the zone of inhibition around Ceftazidime/Clavulanic acid Disc

All the suspected ESBL were also subjected for comparison to Double disc synergy test (DDST)

Double disk synergy test: Ceftriaxone (CTX)-30µg Ceftazidime (CAZ) 30µg Mueller Hinton agar was inoculated with standardized inoculum (corresponding to 0.5 McFarland tube) using sterile cotton swab. Amoxyclav (20µg amoxycillin and 10µg of clavulanic acid- AMC) disc was placed in the centre of the plate and test discs of 3rd generation cephalosporins of cefotaxime-CTX 30µg and ceftazidime- CAZ 30µg discs were placed at 20 mm distance from the Amoxyclav disc. The plate was incubated aerobically overnight at 37°C. ESBL production was considered positive if the zone of inhibition around the test discs increased towards the Amoxyclav disc or neither disc was inhibitory alone but bacterial growth was inhibited where the two antibiotics diffuse together (7,8,9,11)

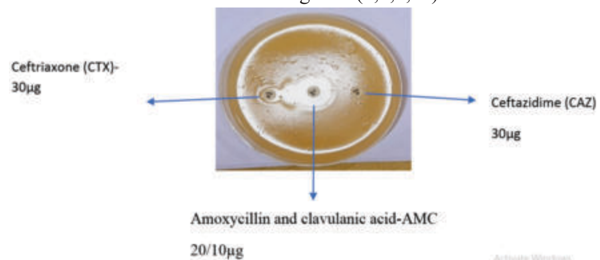

FIGURE 2:
Organism showing enhanced zone of inhibition between ceftazidime and cefotaxime and Amoxicillin/clavulanic acid containing disc indicating ESBL production.

RESULTS:
This study was conducted at Government general hospital, Kakinada and Rangaraya medical college from January 2022 to May 2022, 672 pus sample isolates were collected from different wards.n

Table 1: Showing Gender wise distribution of total number of samples (n=672)

SEX	NUMBER	PERCENTAGE
MALE	368	54.7
FEMALE	304	45.2
TOTAL	672	100

In 672 pus samples 368 (54.7%) highest isolates were in males followed by females 304 (45.2%)

Table-2 : Total number of isolates from different wards (n=672)

ORGANISM	NUMBER	PERCENTAGE
Gram negative Bacilli	485	72.06%
Gram positive Cocci	69	10%
Sterile	118	18%
Total	672	100 %

Out of 672 pus samples, bacterial isolates were 485(72.06%)Gram - negative bacilli, 69(10%) Gram positive cocci and 118(18%) were sterile

Table -3 Different Gram-negative bacilli isolated from pus samples, (n=485)

ISOLATES	FREQUENCY	PERCENTAGE
Klebsiella	154	31.7%
Escherichia coil	122	25.15%
Pseudomonas	80	16.4%
Proteus	72	15%
Citrobacter	57	12%
Total	485	100%

In these 485 Gram-negative bacilli the most common organism was Klebsiella species 154 (31.7%), followed by Escherichia coli 122(25.15%), Pseudomonas 80(16.4%), Proteus 72(15%), Citrobacter 57(12%).

Table-4: ESBL production among Gram-negative bacilli (n=485):

ISOLATES	ESBL Positive	ESBL Negative	Total
Klebsiella sp	84(17.2%)	70(14.4%)	154(31.75%)
Escherichia Coil	62(13%)	60(12.3%)	122(25.15%)
Pseudomonas sp	24(5%)	56(11.5%)	80(16.49%)
Proteus Sp	17(4%)	55(11.3%)	72 (14.84%)
Citrobacter sp	09(2%)	48(10%)	57(11.7%)
TOTAL	196(40.3%)	289(59.4%)	485(100%)
The chi square statistic is 44.6542, the p-Value is <0.00001, result is SIGNIFICANT at p<0.05			

Among the 485 Gram-negative bacilli,196 (40.3%) were ESBL producers and the most common ESBL producer from Enterobacteriaceae is Klebsiella sp 84(17.2%), followed by Escherichia coli 62 (13%), Proteus sp 17(4%) and Citrobacter sp 9(2%) and in non-Fermenters is Pseudomonas sp 24(5%). The p-value is 0.00001, significant among ESBL producers(p<0.05).

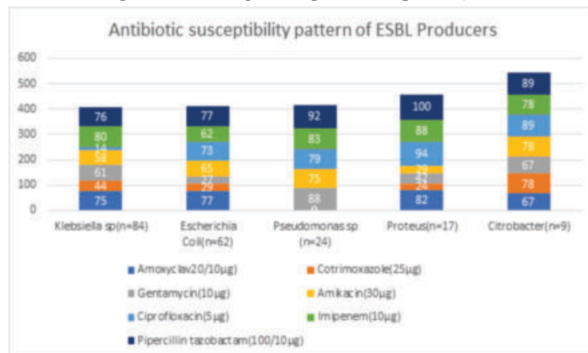

Chart 1: Antibiotic susceptibility pattern of ESBL producers (n=196)

Of all the 196 ESBL producer, Gram-negative bacilli showed most resistant (100%) to antibiotic Ampicillin, ceftriaxone and ceftazidime. Most sensitive to Amoxyclav, Imipenem and Piperacillin-tazobactam.

Table 5: Showing comparison between Phenotypic confirmatory test positive (PCT) and Double disk synergy test (DDST) on detection of ESBL producers (n=196).

Organism	Phenotypic confirmatory test positive (PCT)	Double disk synergy test (DDST)
Klebsiella Spp n =84	82/84(98%)	63/84(75%)
Escherichia coli Spp n =62	50/62(81%)	48/62(77.4%)
Pseudomonas Spp n = 24	20/24(83%)	16/24(66%)
Proteus Spp n =17	15/17(88.2%)	14/17(82.3%)
Citrobacter Spp n =09	7/9(78%)	5/9(55%)
Total (n=196)	174(88.7%)	146(74.4%)

Out of 196 ESBL producers, Phenotypic confirmatory test positive (PCT) were 174(88.7%) and Double disk synergy test were 146(74.4%)

The Phenotypic confirmatory test (PCT) showing chi square statistic is 12.5165 ,the p-value is <0.013897 and the result is SIGNIFICANT p<0.05.

DISCUSSION:

The spread of ESBL producing organisms have been found in a wide range of Gram-negative bacilli, due to frequent usage of antibiotics in both nosocomial and community acquired infections, however, the vast majority of strains expressing these enzymes belong to the family Enterobacteriaceae.The high prevalence of ESBL producing organisms among the clinical isolates of pus samples varies significantly all over the world[12].

The present study was about the comparison of the Double disc synergy test (DDST) and Phenotypic confirmation method (PCT) for ESBL detection and their antibiotic Susceptibility pattern, in clinical isolates of pus samples, which were nosocomial associating bacteria harboring ESBL among the patients of Government General Hospital, Kakinada.

In this study, out of 672 pus samples were collected. From these samples, culture-positive bacterial isolates were 554(82.4%), our study is correlated with the study, of Mita D wadekar14 (85.5%) were positive cultures isolated from pus samples. In our study Gram-negative bacilli were 485 (72.06%), Gram-positive cocci were 69(10%) and 118 (18%) were sterile, our findings are correlated with the studies of Kankadurgamba (15), showed Gram-negative bacilli were 72.2%, Gram-positive cocci were 27.2% and in another study by Rugira (16), showed Gram-negative bacilli were 76.7% and Gram-positive cocci 20%.

In our study, based on CLSI guidelines11, we found that 196(40.3%) suspected ESBLs producers from 485 Gram-negative bacilli isolates. By using third-generation cephalosporins (ceftriaxone-30µg, ceftazidime-30µg) for the screening of ESBL, and our findings are closely related to that of

Table 6:

Author	Year	Prevalence of ESBL
Snehal Mayanand deshmukhe23	2022	53%
Kankadurgamba (15)	2021	38.2%
Madhavi & Hanumanthappa (21)	2021	38.8%
Prateet Kaur (17)	2019	49.8%
Ashish Khanna(16)	2016	39.6%
Present study	2022	40.3%

Out of 196(40.3%) ESBL producers Gram-negative bacilli isolated were Klebsiella spp.(17%), showed the maximum ESBL production(17%) followed by Escherichia coli (13%), pseudomonas(5%), proteus(4%) and Citrobacter (2%) ,was similar to study by Vemula Sarojamma, Vadde Ramakrishna19 in which ESBLs were predominantly present in Klebsiella(17%).other studies like Ashish Khanna, Menka Khanna showed that ESBL predominately seen in klebsiella species(22.2%) followed by Escherichia coli (46.2%),pseudomonas(14.3%),Proteus and Citrobacter. Mathur et al, Klebsiella pneumonia (73%), and Escherichia Coli (62%) were reported ESBL producers.

These 196(40.3%) screening ESBL producers isolates were subjected to the confirmatory tests, 174 (88.7%) were confirmed by phenotypic confirmatory test (PCT) and 146(74.4%) by Double Disc Synergy test (DDST), these findings are greater than the study of Dhandapany Senthil20 detected ESBL producers 84.5% by PCT whereas 61.9% by DDST another studies of Abu Hena Md Saiful Karim Chowdhury7 shown that Phenotypic Confirmatory Test (PCT) detected 62.68% and Double Disc Synergy Test (DDST) detected 52.11%.

Table 7:

Author	Prevalence of PCT and DDST
Dhandapany Senthil	PCT-84.5%, DDST-61.9%
Abu Hena Md Saiful	PCT-62.68%,DDST-52.11%
Srender Kaur	PCT-56.4%,DDST-35.5%
Present Study	PCT-88.7%,DDST- 74.4%

By this comparison found PCCDT to be more effective in detecting ESBL than DDST[(20)].The PCDDT test was compared with DDST and it was found to be an inexpensive alternative for the DDST, for the detection of ESBL producers[22]. The DDST lacks sensitivity because of the problem of optimal disc space and the correct storage of the clavulanic acid containing discs. In currently testing the sensitivity for ceftazidime by using the disc diffusion test and it required only one disc to be added to the sensitivity plate by PCDDT and would screen all Gram-negative bacilli in the diagnostic laboratory for ESBL production. This method is technically simple and inexpensive[7].

In present study, that majority of the Gram-negative bacilli showed resistance to Ampicillin and third generation cephalosporins. Most of them shown susceptibility to Imipenem, Meropenem, AMC (Amoxyclav and clavulanic acid) and CTZ/Cl (ceftazidime and clavulanic acid). Which is correlate with a study of Deepali Gupta (2), showed that Gram negative bacilli are susceptibility to CTZ/CL, Imipenem and Meropenem. Therefore, Imipenem's, Piperacillin /Tazobactam (PIT) is the most active drug for the treatment of infections which are caused by ESBL producers.

ESBL producing organisms are the most common nosocomial pathogens. It is important to detect and treat them to control hospital infections. Carbapenems are the most active antimicrobial agents used to treat the infections, which are caused by the ESBL producing organisms.

CONCLUSION:

Existing of extended spectrum β lactamases (ESBL) in bacteria and Indiscriminative use of 3rd generation cephalosporins and monobactams creates a serious problem in therapeutic conditions, which should be avoided by, the detection of ESBL strains as, they become resistant to routinely using antibiotics and they also transfer the gene to other strains.

So, using of only one disc combination might fail to detect ESBL production, so, the inclusion of more than one indicator drugs in the screening tests is recommended. Phenotypic confirmatory test is better than that the double disc synergy test (DDST) because it is simple and cost-effective method for detection of ESBLs.It is essential to report ESBL production along with the routine susceptibility testing, that will help the clinician in prescribing appropriate antibiotics.

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