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Phenotypic and Molecular Characterization of ESBL sproducing Enterobacteriaceaein A Tertiary Care Hospital

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Abstract
Background :*There is an alarming magnitude of antibiotic resistance, production of Extended-Spectrum β-Lactamases (ESBLs) is a significant resistance-mechanism among Gram Negative bacteria posing challenge to the most coveted wellbeing of man. The Present study was undertaken to detect the prevalence of ESBLs producers, their pedantic antibiogram, and their genes, bla CTX-M, bla TEM &bla SHV by Multiplex PCR.*
Methodology*:In this prospective study, 240 non repetitive Isolates of Enterobacteriaceae from various samples of different departments of Gandhi Hospital were processed for the presence of ESBL by Phenotypic screening, Jarlier DDST, Phenotypic confirmatory test (CLSI guidelines),Vitek2 System, Multiplex PCR for Molecular characterization over a period of 1½ years in the Department of Microbiology Gandhi Medical College and Hospital,Secunderabad,Telangana.*
Results: *Out 0f 240 Gram negative isolates 35.8% were phenotypically confirmed ESBLs producers. OP samples yielded 49/86(56.98%), whereas IP 37/86 (43.02%)(P value 0.0327 statistically significant).Medical and Surgical ICUs yielded 50%, Predominant organism isolated was E.coli 61.62%. All the isolates were 100% resistant to 3rd generation Cephalosporins, 100% Sensitive to Carbapenems, Genotypic characterization was detected in 78 Isolates. Single gene was found in 73.08%. Single Predominant gene was blaCTX-M 71.9%, commonest multiple genes combination was CTX-M&SHV12.82%.*
Conclusion:*Our study confirms global trend of resistance to beta-lactam antibiotics. ESBL strains are usually multi-drug resistant.An appropriate and judicious antibiotic use may lead to withdrawal of the selective pressure.*
Keywords:*ESBLs, DDST, PCT, VITEK 2, CTX-M, TEM, SHV*

I. Introduction

The rapid and irrepressible increase in antimicrobial resistance more so the ESBLs production by Enterobacteriaceae was a worrying global health concern, posing unique challenges to clinical microbiologists, clinicians, infection control professionals and antibacterial-discovery scientists.β-Lactams are safest and most frequently prescribed antimicrobial agents all over the world in treating Gram positive and Gram negative infections[1].

Betalactamases were numerous and the genes encoding them mutate continuously in response to heavy pressure of antibiotic use.They have spread threateningly in many regions of the world creating serious problem to the currently available antibiotic armory.Being Plasmid mediated, it facilitated the dissemination of resistance not only to β lactams but also to other commonly used antibiotics such as fluoroquinolones and aminoglycosides.[2]Quinolone antibiotics were potent promoters of ESBL transmission.Prevalence of ESBLsin United Statesamong *E. coli* ranges from 0 to 25% (NNIS)[3].In Japan <0.1%[4], in Korea 4.8%, ,in Taiwan8.5%and up to 12% in Hong Kong.[5]In India various authors reported the prevalence of ESBLs ranging from4% to 83%.[6]

The first beta-lactamase TEM a plasmid-mediated enzyme named after the patient Temnonierafrom whom it was discovered in Athens of Greece in 1960s,now 175 different TEM enzymes and 127 SHV(Sulfhydryl variable) enzymes exist (www.lahey.org/studies/). Up to 20% of the ampicillin resistance in K pneumoniawas due to SHV-1. In 1989Bauernfeindet al[7]from Germany reported CTX-M-1(Cefotaximase). It had spread globally,currently more than 80 CTX-M enzymes were known.The initial CTX-M arose in the nosocomial setting and spread to the community.The reporting of ESBL producers help the clinicians to select the appropriate antibiotics for the treatment and to take proper precautions to prevent the spread. Some ESBLs may not reach a level to be detected by disk diffusion, but result in treatment failure,identifying the exact ESBL subtype was possible only by molecular detection methods. Though so many types of ESBLs exist majority of the ESBLs were derivatives of TEM, SHV or CTX-M enzymes. Judicious usage of extended-spectrum cephalosporins, periodic surveillance of antibiotic resistance patterns for formulating Antibiotic Policy, and efforts to decrease empirical antibiotic therapy prevent spread and outbreaks of ESBL producing bacteria

II. Aims

1)To determine the prevalence of extended spectrum beta lactamases (ESBLs) among clinical isolates of Enterobactereaceae. 2) To determine their pedantic antibiogram, and Minimum Inhibitory Concentration (MIC) of Antibiotics by Vitek 2 System,3)To detect main ESBL encoding genes such as CTX-M, SHV and TEM by Multiplex PCR (M-PCR) in Gandhi hospital, Telangana.

III. Materials And Methods

A total of 240 non repetitive, clinical isolates of Enterobacteriaceae from a variety of clinical samples from Out-patients and In-patients of Gandhi Hospital were processed in the Department of Microbiology laboratory over a period of 1 ½ years (April 2013 - September 2014) The Isolates were identified, both by conventional Method (Mackie and McCartney Practical Microbiology[8]**,** Koneman`s color atlasand textbook of diagnostic microbiology Diagnostic microbiology[9]**)** and Vitek 2 System [using Identification GN13 cards; AST (Antibiotic Susceptibility Testing) cards bio Merieux, Durham, NC]

The Isolates were screened for possible ESBL production by Screening tests, Doubledisc synergy test (DDST) and the Phenotypic Confirmatory Test (PCT) as per the guidelines issued by Clinical Laboratory standards Institute 2013.[10]

Co-resistance to fluoro-quinolones, aminoglycosides, trimethoprim–sulphamethoxazole, and beta lactamase inhibitor combinations and susceptibility to carbapanems were determined by disc diffusion method, control strains used were

Positive control : ESBL-producing organism (*Klebsiellapneumoniae*ATCC 700603)
Negative control: Non-ESBL-producing organism (*Escherichia coli* ATCC 25922)

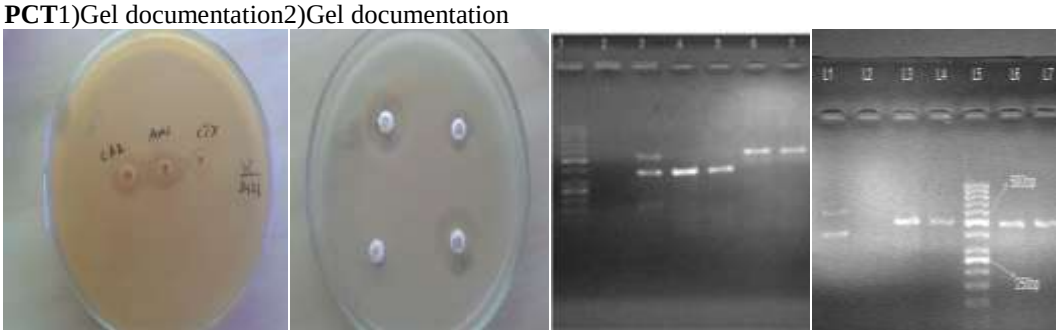
The 86 ESBL producers confirmed by PCT were subjected for detection of genes encoding Beta Lactamases by multiplex PCR amplification using primer sets for blaCTX-M, blaTEM, blaSHV genes, according to the methodology described in the Sambrook and Russell[11]and referred in an article by Meeta Sharma et al[12](2012) for primers. **Molecular Method**: A)DNA extractionwas done by boilingethod,B)DNAamplification-Multiplex PCRC) Gel electrophoresisand Visualization of PCR products under UV lights by trans-illuminator. The Master mixwas prepared as follows: 2.5µL of PCR buffer, 2.5µLof10mM dNTP mix, 1µL of Taq DNA Polymerase, 22µL of RNase free water and 2µL of each of the forward and reverse primers.

CTX-M beta-lactamase: 560 bp
Forward Primer : 5'- GAAGGTCATCAAGAAGGTGCG -3'
Reverse primer :5'- GCATTGCCACGCTTTTCATAG- 3'
TEM beta-lactamase: 459bp
Forward Primer : 5' - GAGACAATAACCCTGGTAAAT- 3'
Revers primer :5'- AGAAGTAAGTTGGCAGCAGTG- 3'
SHV beta-lactamase: 383bp
Forward Primer : 5'-GTCAGCGAAAAACACCTTGCC- 3'
Revers primer :5'-GTCTTA`TCGGCGATAAACCAG- 3'

DNA amplification in thermal cycler: An initial denaturation step at 95°C for 5min followed by 35 cycles of denaturation at 95ºc for 30 sec, annealing at 60ºc for 30sec, extension at 72ºc for 2 min ending with a final extension step at 72°C for 10 min and followed by a hold at 4°C.

Agarose gel electrophoresis:Was done for The PCR products with 1.8% agarose gel using suitable molecular weight markers.

DDST PCT1)Gel documentation2)Gel documentation



1)Gel documentation of Multiplex PCR analysis showing **2)**Gel documentation of Multiplex PCR

Lane-1 ,50bp DNA ladder,Lane 2 Negative controlanalysisthe presence of ESBL genes.

Lane3positive control SHV,CTX-M Showing the presence of ESBL genesLane-1 Positive Control, Lane 2 Negative Control
Lane -4 and 5-SHV ,Lane -6 and 7 CTX-M Lane -3 ,4,6,and7 TEM, Lane 5 DNA ladder50 bp
EPI INFO 2000 version statistical soft ware package was used for statistical analysis. Chi-Square test was applied to test whether differences between values were significant. P values <0.05 was considered as statistically significant. P>0.05 insignificant.

IV. Results

Out of 240 Gram Negative isolates, 35.83% were ESBLs producers by PCT and by Vitek 2 system, molecular characterization was found among 32.5%. More number of Isolates were42.1% among 41-60 years of age group, followed by >60yrs (36.5%), 21-40(26.9%) and 1-20(18.18%). Males were 38.83%, Females 33.5% (Table1).More number of isolates were fromOP49/86(56.98%) whereas IP 37/86 (43.02%)(Table2).p value 0.0327 statistically significant. Medical and Surgical ICUs yielded 50% followed by ICCU 47%, Ortho 45%, OBG 25%, Surgery 20%, Medicine 16.66% and least from ENT 6.6%(Table 3).Higher frequency among Pus samples 41.8% followed by Urine 36.25%, Sputum 33.3% lowest from blood 15%(Table 4).Escherichia coliwas the most common isolate 61.63% followed by klebsiella pneumonia30.23%, Proteus species 5.81%, Citrobacter 2.33%. All the isolates were Resistant (100%)toCeftazidime MIC >=4, Cefotaxime, Ceftrioxone MIC>=8, Cefuroxime MIC>=64 andAztreonum MIC>=2, Sensitive (100%) to Imipenem, Meropenem, Doripenem and Ertapenem MIC rangewas<=0.12 to <0.5.

Among theE.coli isolates highest resistance was observed with Nalidixic acid92.45%,followedby Amoxicillin/clavulanicacid 88.67%, Trimethoprim/Sulfmethaxazole84.9%, Ciprofloxacin 81.13%, Levofloxacin 69.81%,Minocycline 67.92% and sensitivity was high with Cefperazone/sulbactam96.23% followed by Piperacillin/Tazobactam79.24%.Most prevalent gene was CTX-M 60.37% followed by TEM11.32%,SHV3.77%,CTX-M+TEM9.4%,CTX-M+ SHV11.32%, not characterized were 3.77 %.
Among the Klebsiella isolates resistance was highest 92.3%, with Amoxicillin/clavulanic acid and Ciprofloxacin followed byNalidixic acid 88.46%, Trimethoprim /sulfmethaxazole76.92%, Levofloxacin76.9% and highest sensitivity was observed with Cefperazone/sulbactam 96.16% followed by Gentamycin 69.24%Piperacillin/ Tazobactam 65.39%,Amikacin61.54%(Table 5).Out of 86 phenotypically confirmed ESBLs 78 were genotypicallyconfirmed(Table 6).

Single gene was found in 73.08%, multiple genes were found in 26.92%(Table 7). Most prevalent gene was CTX-M 34.61%, followed by TEM 15.38%, SHV11.53%, multiple genes were TEM+ SHV 19.23% ,CTX-M+SHV15.38%, not characterized was 3.84%.
Proteus species(20%), and Citrobacter(8%) were isolated from surgery department.Among the Proteus SHV(20%) and TEM+SHV(20%), 60% were not characterized, Citrobacter isolates 100% were not characterized(Table 8)..

V. Discussion

The emergence of ESBL-producing bacteria had been strikingly increasing worldwide, and still considered as threat since they were transferred by plasmid which can be easily transmitted between the species.[13]The therapeutic options for these infections became increasingly narrowed down of the pipeline increasing morbidity and mortality.Hence continuous monitoring systems and effective infection control measures were absolutely necessary.

The present study revealed 35.8% prevalence of ESBL producers which correlated with N Fam et al[13] from Egypt 35.7%, slightly lesser than NeemaShashwatiet al[14]from Mumbai 48.27%, Meeta Sharma[12] from Jaipur reported 52.49%,Rodrigues *C* et al[15] fromMumbai showed 53%whereas R.Canton et al[16] from Europe and Goosens et al[17]found 1-12% to as high as 39-47% in Russia, Poland and Turkey.The wide variation in the prevalence was probably due to differences in the risk factors and the excessive use of cephalosporins, and a high rate of patient transfer from the peripheral centerswho received prior multiple antimicrobial treatments with high end antibiotics Mathur P et al[18]

The study showed high prevalence of the ESBL producers among 41-60 years age group (46.51%) which was correlating with MetriBasvaraj et al[19]41-60yrs, Lautenbach et al[20]54yrs median age,This may be because more number of patients were hospitalized in that age group and had other risk factors like Diabetes Mellitus.Whereas GopalKashyapet al[21]showed20-40yrs age group (43 %)as UTI was more common in middle age group.

There was slight male preponderance38.83%this was correlating with Lautenbach et al,[20]Nema Shashwati,[14]Nibedita Das *et al.*[22]Whereas femalepreponderance was noticed byKiratisin et al[23]from Thailand and MetriBasvarajet al[19]from Bijapur as number of female patients suffering from UTI with ESBL producers were more.

More ESBL isolates were from Out-Patient departments of various disciplines 49/86(56.98%) whereas IP 37/86 (43.02%) (P value 0.0327 statistically significant.),which was in accordance withShakti Rath et al[24] from Bhubaneswar OP 56.31%, IP 43.69%, slightly lower than GopalKashyap et al[21]OP 65.6%Whereas VemulaS et al[25]from Kurnool, reported more from IP departments 60%. In the beginning ESBLs were a hospital based crisis but now becoming more common among community acquired isolates, this should alert the physician.Medical and surgical ICUs yielded more number of isolates 50%, followed by the ICCU45% this was comparable withreports by Varsha Gupta et al[26]from Chandigarh and FaustineNdugulile et al[27] from Tanzania, Marra[28] et al from Brazil., and Mathur et[18] al 79% from AIIMS New Delhi,whereas MethriBasavarajuet al[19]showed more from ICCU.This could be due to the prolonged hospital stay, indwellingCanulas, catheters, endotracheal or nasogastric tubes, gastrostomies or tracheostomies and due to severe illness.

ESBL distribution was more among pus samples 41.8%, which was correlating with Dasgupta Rubin et al[29] from Dehradun, ManinderKaur et al[30] fromAmritsar andVarsha Gupta et al[26]from Chandigarh Whereas GauravDalela et al[31] and R.Eshwar Singh et al[32] reported urine and Marra[28] from blood.Majorityof ESBL positive isolates were E. coli 61.6% this wasslightly lesser than that ofR.Eshwar Singh et al[32]67% from Devangere,Pathak A[33] from Ujjain 69%, in a reportfrom Pondicherry 81% Slightly higher compared to NemaShashwati et al[16]50.14%.Rudresh et al[34]40.2%.The percentage of Klebsiellapneumoniae 30.2%, was in accordance with M.Karagaret al[35] 30.55%from Iran.The percentage of Proteus spp 5.81% was correlating withVidyaPaiet al[36]5.5% from Mangalore. The percentage of Citrobacterspp 2.32% was less comparedChoiSet al[37] who reported 4.9%from Korea, very much less compared to Shresthaetal[38]16.6%fromNepal.

Of the 240 isolates ESBL producers by using the PCT were 86(100%), DDST 45.34%. Emery[39]also found DDST could not detect all ESBL-producing isolates. PCT was technically simple cost effective, and 100% in concordance with MIC of Vitek 2 System report.BradfordPA[1] explained that false-positives may occur if the isolate lacks ESBL but produces an excess of TEM-1 or SHV-1on the other hand, isolates harboring both ESBLs and AmpC-type β-lactamases may result in false-negative results.

VITEK 2 system a reliable timesaving tool for routine identification of ESBL-producing strains.All the isolates 86were sensitive100% to Carbapenamsasthese antibiotics were highly stable to β-lactamase activity they remain the only choice of treatment for infections by ESBLs producers.All the Isolates were 100% resistant to 3rd generation Cephalosporins&Aztreonum, which was in accordance with ManoharanAet al[40], from Vellore,Sanjeev Kumar et al[41] from Udaipur, Akbar M. Rafay et a[42] from Oman. R.Bonnetet al[43]Nema Shashwati[16] found Most of them were ceftazidimases, and only a few are cefotaximases.This high degree of drug resistance could be due to the availability of drugs to patients with or without any prescription and discontinuing them in the middle of the course.

E.coli isolates showed highest resistance toNalidixic acid 92.45%, followed by Amoxicillin/clavulanicacid 88.67% Trimethoprim/sulfmethaxazole84.9%, Ciprofloxacin 81.13%,Levofloxacin69.81%,Minocycline 67.92%, Nitrofurantoin 60% and sensitivity was 100% for Carbapenam,96.23% for Cefperazone/sulbactam followed by Piperacillin/Tazobactam83.02% which was nearer to the reports of MeetaSarma et al from Jaipur,[12]Wong-Beringer[44]et al.from Pakistan.The Klebsiella isolates were highly resistant to Amoxicillin&clavulanicacid and Ciprofloxacin92.3% followed byNalidixic acid 88.46% Trimethoprim&sulfmethaxozole, Levofloxacin 76.92%, sensitive was 100%for Carbapenam96.16% for Cefperazone/sulbactamfollowed byGentamycin 69.24%, Piperacillin/ Tazobactam 65.39%, Amikacin 61.54%.. Which was near toMeetha Sharma[12] from Jaipur, Ullah *et al*[45]from Pakistan

Jacoby[46] explained, that this may be due to the occurrence of genes encoding resistance to aminoglycoside, trimethoprim–Sulfomethoxazole, and quinolones on the same plasmid that encodes for ESBL production. Martínez-Martínez[47] and colleagues in their analysis found porin loss among the ESBL producing K.pneumoniae also showed active efflux of quinolones, Energy dependent accumulation of Norfloxacin.

Out of 86 phenotypically confirmed ESBLs 78 showed genotypic characterization. More number of isolates expressed single genes 73.71% multiple genes were 26.29%. This was nearly correlating withMeeta Sharma[12] from Jaipur, Mehdi Kargar[35] from Iran. Whereas Kaftandzievaet al[48]from Skopze reported mixed genes.

Among the ESBLs Producing E.coliCTX-M-60.37% TEM-11.32% SHV-3.77% CTX-M+TEM 9.4%, CTX-M+SHV 11.32%, isolatesnot characterizedwere3.77% nearly correlating withManinderKaur[27]CTX-M genes 59.32%, CTX-M+TEM 8.4%. Meeta Sharma et al[12] from Jaipur CTX-M80%,Ho.P.L[49] observed UTI due to CTX-M ESBL produce E. coli strains, A Manoharanet al[40] observed TEM and CTX-M were predominant. Most prevalent gene among Klebsiella was CTX-M 34.61%, followed by TEM+ SHV 19.23%, TEM 15.38, CTX-M + SHV15.38%, SHV11.53%, not characterizedwas3.84%. Slightly lesser thanFeizabadi MMet al[50] reported bla(CTX-M-I) 46.51%. Mubarak et al[51] from UAE reported the emergence and dissemination of CTX-M-15 as87%among E. coli and K. pneumoniae CTX-M enzymes are now endemic over a wide geographic area, Most strains producing CTX-M enzymes seem to be implicated in nosocomial infections.

James H. Jorgensen[52]found CTX-M prevalent globally Livermore et al[53] R.Bonnet[54]found CTX-M gene replacing TEM and SHV types in many European and Asian countries.Mathai D et al[55]observedCTX-M as

Predominant gene in India. The easy dissemination, extraordinary spread of the CTX-M enzymes deserving an uncontrolled pandemic scenario was due to the resistance determinants and the frequency of transfer in the range of 10^{-7} to 10^{-2} per donor cell*bla*$_{CTX-M}$ harboring plasmids.

Table No.1 Sex wise distribution of ESBL producers

Sex	Total n=240	ESBL Positive (n=86)	ESBL Positive %
Male	103	40	38.8
Female	137	46	33.5

Table No.2 Distribution of ESBL producers in O P and I P Patients

Total no N=240	Total I.P N=136		Total O.P N=104	
Total ESBL positive N= 86	ESBL positive N=37	ESBL negative N= 99	ESBL positive N=49	ESBL negative N= 55

Table No.3Distibution of ESBL Producers among various Departments

Department	Total Isolates	Totalno ESBLS= 86	*Escherichia coli* n =53	*Klebsiella pneumoniae.* n=26	*Proteus SPP* n=5	Citrobacter *Freundii*. n=2
MICU	40	20(50%)	15(28.3%)	5(19.23%)	-	-
SICU	50	25(50%)	12(22.6%)	6(23.07%)	5 (20%)	2 (8%)
ICCU	32	15(47%)	11(20.7%)	4(15.3%)	-	-
Ortho	20	9(45%)	4(7.54%)	5(19.23%)		
OBG	25	5(20%)	3(5.6%)	2(7.69%)	-	-
Medicine	25	5(20%)	3(5.6%)	2(7.69%)	-	-
Surgery	25	5(20%)	4(7.54%)	1(3.84%)	-	-
ENT	30	2(6.6%)	1(1.88%)	1(3.84%)	-	-

Table No 4 Distributionof ESBL producing organism in various clinical samples

Name of the organism	Pus	Urine	Sputum	Stool	Vaginal swabs	Blood
Escherichia coli n =53	28(52.8)	21(39.62%)	-	2(3.77%)	2(3.77%)	
Klelpneumoniae.n=26	11(42.3)	8(30.76%)	4(15.38%)			3(11.5 %)
*Proteus*Sppn=5	5(100%).					
Citrobacter*freundii*. n=2	2(100%).					

Table No.5 Antimicrobial resistance pattern among ESBL positive isolates

Name of Antibiotic	*Escherichia coli*, N=53			*Klebsiellapneumoniae N=26*			*Proteus spp. N=5*			Citrobacterspp N=2		
	S	I	R	*S*	*I*	*R*	*S*	*I*	*R*	S	I	R
Ceftazidime	-	-	53	-	-	26	-	-	5	-	-	2
Ceftriaxone	-	-	53	-	-	26	-	-	5	-	-	2
Cefuroxime	-	-	53	-	-	26	-	-	5	-	-	2

Cefuroxime axetil	-	-	53	-	-	26	-	-	5	-	-	2
Ampicillin	-	-	53	-	-	26	-	-	5	-	-	2
Amoxiclav	4	2	47	1	1	24	0	0	5	0	0	2
Nalidixic acid	4	-	49	3	-	23			5			2
Trimethoprim/ sulfamethoxazole	8	-	45	6	-	20	-	-	5	-	-	2
Ciprofloxacin	10	-	43	2		24	2	-	3	1	-	1
Levofloxacin	14	2	37	4	2	20	3	-	2	1	-	1
Minocycline	16	1	36	11	2	13	-	-	5	-	-	2
Nitrofurantoin	26	-	27	11	-	15	-	-	5	-	-	2
Gentamicin	25	1	27	17	1	8	2	-	3	-	-	2
Tigecycline	31	-	22	11	-	15	-	-	5	-	-	2
Amikacin	33	-	20	16	-	10	3	-	2	1	-	1
Piperacillin /Tazobactum	42	2	9	16	1	9	1	1	3	1	-	1
Cefperazone/sulbactum	50	1	2	15	1	10	3	1	1	2	0	
Imipenem	53	-	-	26	-	-	5	-	-	2	-	-
Doripenem	53	-	-	26	-	-	5	-	-	2	-	-
Meropenem	53	-	-	26	-	-	5	-	-	2	-	-
Ertapenem	53	-	-	26	-	-	5	-	-	2		--

Table No 6 Phenotypic and Genotypic distribution of ESBLs

Total no of samples	Phenotypic confirmatory test positives	Genotype positive	Genotype Negative
240	86	78(90.69%)	8(9.3%)

Table No. 7 Distribution pattern of Single and Multiple genes among ESBL producers

Phenotypic ESBL positives	Genotypic ESBL positives	Single genes	Multiple genes
86	78(90.69%)	57(73.07%)	21(26.92%)

Table No 8 Distribution of ESBL genes among the different isolates

Organism	CTX-M N=41	TEM N=10	SHV N=6	CTXM+ TEM N=5	CTX-M + SHV N=10	TEM+ SHV 6	Not characterized n-8	Total
E.coli	32 60.37%	6 11.32%	2 3.77%	5 9.4%	6 11.32%	-	2 3.77%	53
K.pneumoniae	9 34.61%	4 15.38%	3 11.53%	-	4 15.38%	5 19.23%	1 3.84%	26
Proteus spp	-	-	1 20%	-	-	1 20%	3 60%	5
Citribacter	-	-	-	-	-	-	2 100%	2

VI. Conclusion

ESBL strains are usually multi-drug resistant. Quick detection of these strains in microbiology laboratories is very important,A combination of different tests can be useful for accurate identification so that appropriate treatment is instituted to reduce morbidity and mortality substantially. Data on institutional antibiograms are necessary for formulating antibiotic policy and for Antibiotic Stewardship to control Hospital Acquired Infections(HAI). An appropriate and judicious antibiotic use may lead to withdrawal of the selective pressure.

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