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Prevalence, Antimicrobial Susceptibility Profiling, and Molecular Identification of ESBLs producing Gram Negatives Bacterial isolates from Hospital Settings

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Abstract

This study was inaugurated to check the frequency, antimicrobial sensitivity, and Molecular identification of ESBL-developing Gram-negative bacteria. This descriptive research was conducted at private and public sector hospitals of four Tehsils of District Jhang, Pakistan. The quick increase in extended-spectrum beta-lactamases (ESBLs) cases among foremost Gram-negative microorganisms raises risk and a worldwide concern. A total of 200 samples were composed from an environment of different public and private hospitals. Samples were processed for isolation and identification of gram-negative bacteria by standard microbiological and biochemical characterization. The antibiotic susceptibility testing (AST) was detected by the Kirby-Bauer disk diffusion method while the presence of ESBLs was detected by the double disc diffusion (DDST) test. PCR amplification of isolates was performed to characterize resistant genes, and statistical analysis was performed to check the significance of data. The overall antimicrobial susceptibility of E. coli and K. pneumoniae was (28%), P. aeruginosa (39.5%), A. baumannii (23.2%), P. mirabilis (38.8%), and S. marcescens (55%). However, overall susceptibility to cephalosporins was (14.6%). PCR revealed 46 (47%) isolates confirmed positive for ESBL encoding genes. Among these total positive ESBL genes, *bla*-CTX-M in 26 (56.52%) strains followed by *bla*-TEM in 13(28.26%) strains and *bla*-SHV in 5 (10.86%) strains. Only two genes bla-CTX-M/*bla*-TEM were present in 1 (2.17%) strain. Moreover, three of these genes *bla*-CTX-M/*bla*-TEM/*bla*-SHV combination found only in 1 (2.17%). The prevalence of ESBL-producing isolates has increased day by day. Isolates had a high prevalence of ESBL-encoding bla-CTX-M gene and bla-TEM and bla-SHV were prevalent

Keywords: AST, Multiplex PCR, ESBL, Phenotypic test, Molecular tests, Pakistan

Introduction

The propagation of antimicrobial resistance (AMR) is seen as a severe community health problem all around the world. The accessibility of efficient antimicrobial drugs is critical in the treatment of bacterial illnesses [1]. According to projections, by 2050, nearly 10 million individuals will die each year due to certain diseases caused by antimicrobial resistance bacteria [2]. Antimicrobial resistance is a natural phenomenon interrelated to widespread antibiotic use, overuse, and nonmedical use. [3]. Due to their high resistance rate, Gram-negative bacteria are on top of the antibiotic-resistant microorganisms list to aid in the detection, development, and research of new antimicrobial drugs [4]. The antimicrobial drugs, β-Lactam mainly used to treat serious hospital-acquired diseases due to gram-negative rods [5].

In gram-negative bacteria, the extended-spectrum β-lactamase (ESBLs) production is a leading resistance mechanism to the β-lactam antibiotics which has great importance for the 3rd generation of cephalosporins resistance (e.g., Ceftriaxone, Ceftazidime, and Cefotaxime). For the last 20 years, gram-negative bacterial infections due to ESBL production have arisen as a big issue in many hospital settings [6]. β-lactam ring of antimicrobial drugs can hydrolyze by the beta-lactamase enzymes produced by Enterobacteriaceae. ESBLs enzymes that can hydrolyze and facilitate resistance to penicillin, monobactam, and cephalosporins. Carbapenemase has a comprehensive hydrolyzing action that degrades all β-lactams including carbapenems and β-lactam inhibitors like sulbactam, clavulanic acid, and tazobactam [7]. The ESBL enzyme gene is found on plasmids and evolved from point mutations that alter the active site structure of previously known lactamases [8] [8].

The most prominent β-lactamase genes including CTX-M, TEM, SHV, OXA, VEB, PER, and TLA widened the substrate specificity against ceftriaxone, ceftazidime, and cefotaxime [7, 8]. These genes are predominantly found in Escherichia coli and Klebsiella spp [9]. While Pseudomonas spp. and Acinetobacter spp. predominantly contains OXA genes [10]. Furthermore, certain clinical microorganisms have β-lactam genes more than once [11]. Multiplex PCR-based recognition systems are now a commonly utilized tool for epidemiological surveys due to the diversity of such enzymes [11, 12]. Medically significant hospital-acquired infectious pathogens, antibiotic-resistant bacteria, and repositories of resistance genes are normally present on numerous surfaces within hospitals (e.g., workplaces, housekeeping surfaces, medical equipment, and lobby (furniture) [13]. Antimicrobial-resistant strains are becoming more common in hospitals, especially in poor countries, and pose a challenge to the management of hospital-acquired infections. [14, 15].

In Pakistan in the last few decades, a continuous rise in ESBL-associated infections has been seen [16-18]. Antibiotic resistance in bacteria is affected by unproductive national or international surveillance systems, ineffective disease control agencies, a shortage of facilities, and ineffective investigative procedures. [11, 17, 19]. Antimicrobial sensible usage and better disease control approaches must be the main concern. Despite the current global rise of ESBL isolates from hospitals, their transmission has received little attention, particularly in inanimate hospital environments in Pakistan, particularly in areas where there is no true antimicrobial resistance policy. Therefore, this research aimed to determine the proportion of environmental contamination of ESBL-producing Gram-negative bacteria and antimicrobial susceptibility profiling in Pakistan. Moreover, the study provides updates on the genotyping of the ESBL-producing gram-negative bacteria in the studied area.

Experimental section

Study design and sampling location

This descriptive study was conducted at private and public sector hospitals of four Tehsils of District Jhang from February to May 2020. The samples were collected from the intensive care unit (ICU), surgical ward, medical ward emergency ward, and outdoor patient and from different medical equipment and surgical instruments.

Sample Collection

A total of 200 samples including 40-pre- and post-operational passive air samples executed according to the 1/1/1 scheme of the settle plate method. Moreover, 160 sterile swabs after being humidified with buffered saline rolled over different Surgical Instruments, Nebulizers, Ventilators, Infusion Sets, Oxygen Mask, Suctions, ECG Sensor, Laryngoscope & Blade, Door Handles, Walls, Theater bed covers, Floor, Sink, Patient beds, Drugs Trolley, Mounting Sleeves and other. These samples were lawn on various selective media such as MacConkey's agar and Eosin Methylene Blue agar for Gram negatives organisms' isolation. The plates were kept at 37°C for 24 hours. The Gram-negative bacteria recognized at the species level through Gram stain and certain biochemical tests like catalase, citrate, coagulase, oxidase, Voges-Proskauer (VP), methyl red, Triple Sugar Iron Agar (TSI), urea, and indole [20, 21].

Antimicrobial susceptibility testing

Antimicrobial Susceptibility test (AST) was accomplished by using different antibiotics (Oxoid, UK) according to the Kirby-Bauer disk diffusion procedure performed on Mueller Hinton agar medium (Oxoid, UK) as elaborated by Clinical and Laboratory Standards Institute (CLSI, 2008). Each bacterial blended pure culture inoculum suspension incubated overnight (0.5 McFarland) smoothly streaked on Mueller Hinton agar medium plates through sterile swabs and applied the antibiotics after 3 3-minute wait. For Gram-negative bacteria, following antibiotics were applied: imipenem (10µg), meropenem (10µg), amoxicillin (10µg), ampicillin (10µg), ciprofloxacin (5µg), levofloxacin (5µg), ceftriaxone (30µg), cefotaxime (30µg), ceftazidime (30µg), cefepime (30µg), cefoxitin (30µg), amikacin (30µg), gentamicin (10µg) and Sulfamethoxazole trimethoprim as recommended by Clinical Laboratory Standards Institute (CLSI) [21].

Phenotypic ESBL-screening

ESBL generation of the isolates was confirmed through a double disk synergy test (DDST) as stated by Jarlier et al. [22]. Overnight incubated culture suspension of isolates with concentration adjusted to 0.5 McFarland standard applied on the Mueller Hinton Agar plate with the help of a sterile cotton swab. The Cefotaxime (30 µg) alone and cefotaxime combined with clavulanic acid (30 µg/ 10 µg) disks were adjusted 20 mm apart on the agar plates. Similarly, the ceftazidime (30 µg) alone and ceftazidime combined with clavulanic acid (30 µg/ 10 µg) disks were positioned 20 mm apart. Same as ceftriaxone (30µg) alone and ceftriaxone combined with clavulanic acid (30 µg/ 10 µg) disks positioned 20 mm apart on the agar. A 5mm zone increase for either antimicrobial drug tested in conjunction with clavulanic acid vs. its zone when tested alone was considered as positive for ESBL formation after overnight incubation at 37°C. The control strains were Escherichia coli ATCC 25922 and Klebsiella pneumonia ATCC 700603 [23].

Molecular Identification

ESBLs were confirmed using phenotypic detection methods and then processed using molecular tools to look for ESBL gene variations. The DNA was extracted by the heat lysis method used to run PCR [24]. In Multiplex PCR, 2µl entire cell lysate DNA for every specimen is used individually in 25µl PCR head mix and primers for amplification as cited [24, 25]. Conditions for PCR amplification were start with denaturation at 95°C for 5 minutes followed by 35 cycles of denaturation at 95°C, annealing at 56°C, extension at 95°C and the final extension was done at 95°C for 10 minutes. PCR product visualized on 1% Agarose gel by electrophoresis. [26].

Table.1. Primer sequences and amplification conditions

Target Gene	Primer	Sequence	Size (bp)	Annealing Temperature ˚C	Reference
bla_{TEM}	TEM-C	ATCAGCAATAAACCAGC	516	56˚C	26
	TEM-H	CCCCGAAGAACGTTTTC			
bla_{SHV}	SHV-fwd.	AGGATTGACTGCCTTTTTG	392	56˚C	26
	SHV-rev	ATTTGCTGATTTCGCTCG			
bla_{CTX-M} group 1/2	CTX-M fwd.	CGTCACGCTGTTGTTAGGAA	780	55˚C	26
	CTX-M rev	ACGGCTTTCTGCCTTAGGTT			

Statistical analysis

The Chi-square test was used to verify the position of the association study variable with ESBL and non-ESBL-producing E. coli, K. pneumoniae, P. aeruginosa, and A. baumannii. A two-tailed p-value of <0.05 was considered significant. The statistical analysis was achieved using SPSS 20.

Results

Distribution of clinical isolates

A total of 49% (n=98/200) ESBL-producing bacteria were found among 200 gram-negative isolates. These comprise 59% (n=45) E. coli, 46% (n=33) K. pneumoniae, 50% (n=18) P. aeruginosa and 33% (n=2) Acinetobacter spp. found as presented in Table 2.

Table.2. Association of ESBLs with type of Specimen

Isolates	Frequency	Positive (%)	Negative (%)	*p*-value
Escherichia coli	76	45 (59.2)	31 (40.8)	0.044
Klebsiella pneumoniae	72	33 (45.8)	39 (54.2)	0.043
Pseudomonas aeruginosa	36	18 (50.0)	18 (50.0)	0.043
Acinetobacter baumannii	6	2 (33.3)	4 (66.7)	0.001
Proteus mirabilis	6	0 (0)	6 (100)	0.001
Serratia marcescens	4	0 (0)	4 (100)	0.001
Total	**200**	**98 (49)**	**102 (51)**	**0.035**

Microbial air contamination index of air samples done for the mean of CFU/m3/h. The lowest contamination was in ICU, the mean value before the operation was 40 CFU/m3/h and 110 CFU/m3/h during the operation. Similarly, emergency ward (180 CFU/m3/h), Operation Theater (210 CFU/m3/h), surgical ward (250CFU/m3/h) and OPD (296 CFU/m3/h) were found contaminated during Operational respectively with p-value <0.05 as represented in Figure.1.

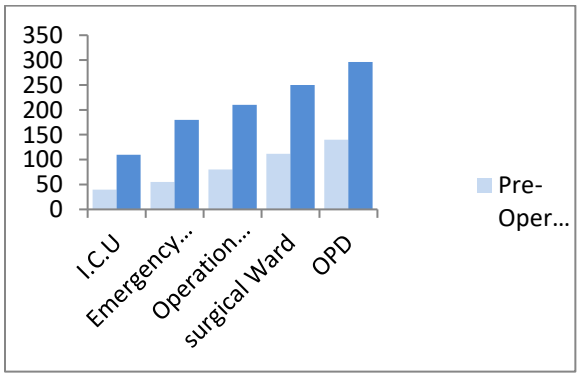


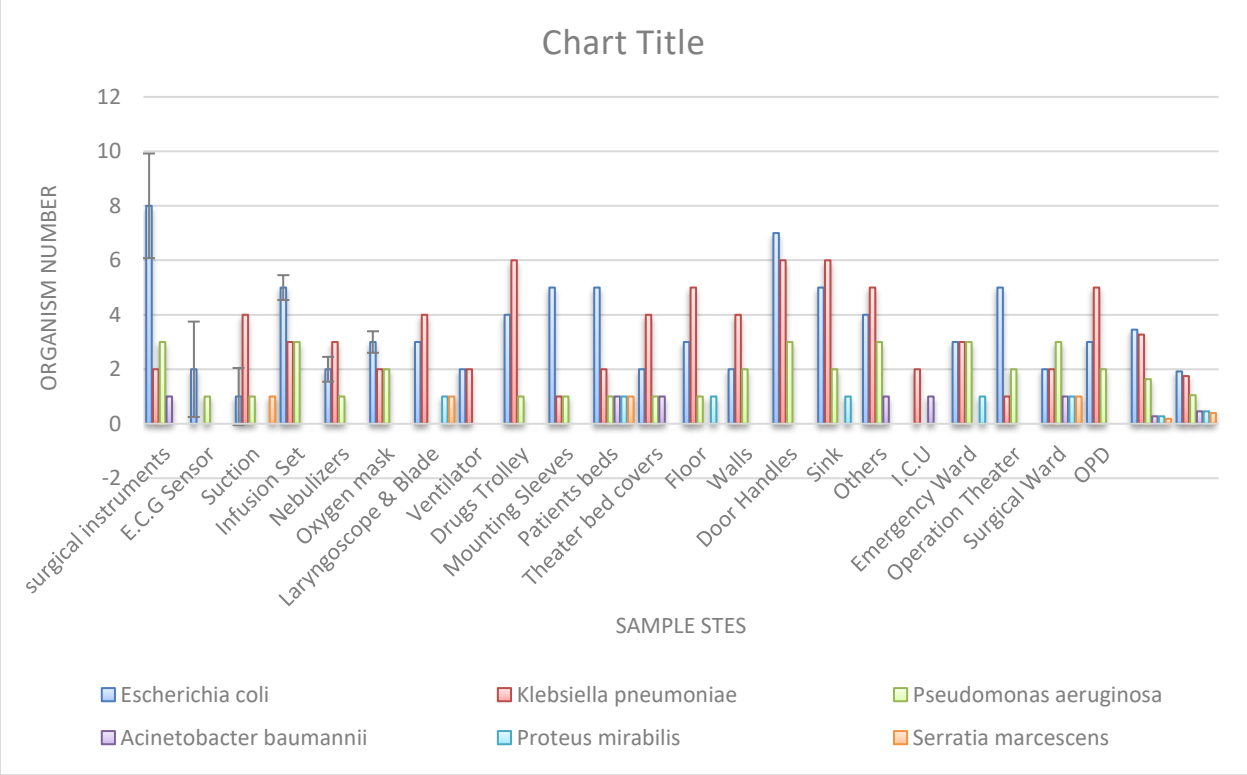
Figure 1: Mean CFU/m3/h values for different sites before and during the operation.

The highest bacterial contaminated samples were obtained from Door handles of different wards followed by Surgical Instruments, Sinks, and beds. Door handles were mostly contaminated with *Escherichia* species (43.75%, 7/16), followed by *Klebsiella* spp., (37.5%, 6/16). Surgical instruments are mainly infected with *Escherichia spp* (57.1%, 8/14). Sinks are mainly colonized by *K. pneumoniae* (50%, 4/8) and *Pseudomonas* spp (25%, 2/8). *Acinetobacter* spp is mainly contaminated patient beds (16.7%, 2/9) and drugs Trolley (54.5%, 6/11) mainly contaminated by *Klebsiella spp*. The lowest contamination samples were obtained for I.C.U wards (1.5%), ECG sensor (1.5%) and Ventilator (2%). Table 3.

Table 3. Distribution of isolated microorganism (n = 200) and their percentages (%) by Type of Specimen and Settings

Sampling Site	E.coli (%)	K. pneumoniae (%)	P. aeruginosa (%)	A. Baumannii (%)	P. mirabilis (%)	S. marcescens (%)	Total (%)
surgical instruments	8 (10.52)	2 (2.77)	3 (8.33)	1 (16.66)	-	-	14(7.0)
E.C.G Sensor	2 (2.63)	-	1 (2.77)	-	-	-	3 (1.5)
Suction	1 (1.31)	4 (5.55)	1 (2.77)	-	-	1 (25.0)	7 (3.5)
Infusion Set	5 (6.57)	3 (4.16)	3 (8.33)	-	-	-	11(5.5)
Nebulizers	2 (2.63)	3 (4.16)	1 (2.77)	-	-	-	6 (3.0)
Oxygen mask	3 (3.94)	2 (2.77)	2 (5.55)	-	-	-	7 (3.5)
Laryngoscope & Blade	3 (3.94)	4 (5.55)	-	-	1 (16.66)	1 (25.0)	9 (4.5)
Ventilator	2 (2.63)	2 (2.77)	-	-	-	-	4 (2.0)
Drugs Trolley	4 (5.26)	6 (8.33)	1 (2.77)	-	-	-	11(5.5)
Mounting Sleeves	5 (6.57)	1 (1.38)	1 (2.77)	-	-	-	7 (3.5)
Patients' beds	5 (6.57)	2 (2.77)	1 (2.77)	1 (16.66)	1 (16.66)	1 (25.0)	11(9.5)
Theatre Bed Cover	2 (2.63)	4 (5.55)	1 (2.77)	1 (16.66)	-	-	8 (4.0)
Floor	3 (3.94)	5 (6.94)	1 (2.77)	-	1 (16.66)	-	10(5.0)
Wall	2 (2.63)	4 (5.55)	2 (5.55)	-	-	-	8 (4.0)
Door Handles	7 (9.21)	6 (8.33)	3 (8.33)	-	-	-	16(8.0)
Sink	5 (6.57)	6 (8.33)	2 (5.55)	-	1 (16.66)	-	14(7.0)
Others	4 (5.26)	5 (6.94)	3 (8.33)	1 (16.66)	-	-	13(6.5)
I.C.U	-	2 (2.77)	-	1 (16.66)	-	-	3 (1.5)

Emergency Ward	3 (3.94)	3 (4.16)	3 (8.33)	-	1 (16.66)	-	10(5.0)
Operation Theater	5 (6.57)	1 (1.38)	2 (5.55)	-	-	-	8 (4.0)
Surgical Ward	2 (2.63)	2 (2.77)	3 (8.33)	1 (16.66)	1 (16.66)	1 (25.0)	10(5.0)
OPD	3 (3.94)	5 (6.94)	2 (5.55)	-	-	-	10(5.0)
Total	76 (38)	72 (36)	36 (18)	6 (3)	6 (3)	4 (2)	200



Antimicrobial susceptibility profiling of Isolated Gram-negative Bacteria

Upon testing different antibiotics including carbapenems, β-lactams, fluoroquinolones and cephalosporins groups *Escherichia coli* exhibited the maximum resistance including ampicillin 66(87%), amoxicillin 64(84%), cefepime 64(84%), ceftriaxone 61(80%), ceftazidime 60(79%) and cefotaxime 60(79%). Low resistance by *Escherichia coli was* documented to imipenem 8(12%) and amikacin 16(21%). Similarly, *K. pneumoniae* presented the maximum resistance to certain antibiotics; Ceftriaxone 69(96%), amoxicillin 63(87%), cefepime 61(85%), sulfamethoxazole trimethoprim 61(85%) and ceftazidime 59(82%). Low resistance level by *K. pneumoniae* recorded to Levofloxacin 21(29), meropenem 22(30%), ciprofloxacin 24(33%) and meropenem 28(39%). In the case of *P. aeruginosa, the* highest resistance level was found against ampicillin 33(92%), ceftazidime 32(89%), cefotaxime

31(86%), ceftriaxone 29(81%), cefoxitin 29(81%) and amoxicillin 28(78%). Low resistance level by *P. aeruginosa* recorded to amikacin 2(5%), imipenem 4(11%), ciprofloxacin 7(20), gentamicin 8(22%), meropenem 8(22%) and levofloxacin 10(28%). *A. baumannii* shows highest resistance level against ampicillin 6(100%), amoxicillin 5(83%), ceftriaxone 5(83%), cefoxitin 5(83%), ceftazidime 5(83%), cefotaxime 5(83%) and sulfamethoxazole trimethoprim 4(67%). Low level of resistance was found against imipenem 1(17%), meropenem 2(33%) and amikacin 2(33%). *P. mirabilis* showed highest resistance against amoxicillin 5(83%), cefoxitin 5(83%), ampicillin 4(66%), ciprofloxacin 4(66%) and levofloxacin 4(66%). Low resistance level against ceftazidime 1(17%), imipenem 2(33%) and meropenem 2(33%). *S. marcescens* showed highest resistance level against cefoxitin 3(75%), meropenem 2(50%) and levofloxacin 2(50%). The lowest resistance level was found against most of the antibiotics including cefotaxime 0(0%) highly effective against *S. marcescens*, imipenem 1(25%), amoxicillin 1(25%), ampicillin 1(25%), ciprofloxacin 1(25%), ceftriaxone 1(25%), ceftazidime 1(25%), cefepime 1(25%), and sulfamethoxazole-trimethoprim 1(25%).

Low-level resistance was recorded for imipenem 38(19%), amikacin 62(31%), levofloxacin 68(34%), gentamicin 72(36%), meropenem 75(38%) and ciprofloxacin 74(41%). Most of the Gram-Negative Bacteria presented maximum resistance the antibiotics, ceftriaxone 167(84%), amoxicillin 166(83%), ampicillin 163(81%), ceftazidime 158(79%), cefepime 152(76%), sulfamethoxazole trimethoprim 148(74%), cefotaxime 146(73%) and cefoxitin 125(63%). Table 4.

Table 4. Percentage of Antibiotic susceptibility among different microorganisms isolated from the Hospital environment.

Isolates	Ptn	IPM	MEM	AML	AMP	CIP	LEV	CRO	FOX	CAZ	CTX	FEP	CN	AK	CXT
E. coli	S (%)	58 (76)	32 (42)	9 (12)	7 (9)	21 (28)	39 (51)	10 (13)	26 (34)	12 (16)	9 (12)	7 (9)	33 (43)	47 (62)	16 (21)
	I (%)	10 (13)	11 (14)	3 (4)	3 (4)	20 (26)	9 (12)	5 (7)	16 (21)	4 (5)	7 (9)	5 (7)	8 (11)	13 (17)	4 (5)
	R (%)	8 (12)	33 (43)	64 (84)	66 (87)	35 (46)	28 (37)	61 (80)	34 (45)	60 (79)	60 (79)	64 (84)	35 (46)	16 (21)	56 (74)
K.pneumoniae	S (%)	43 (60)	36 (50)	7 (10)	13 (18)	40 (56)	41 (57)	2 (3)	13 (18)	10 (14)	14 (19)	8 (11)	42 (58)	29 (40)	8 (11)
	I (%)	7 (10)	8 (11)	2 (3)	6 (8)	8 (11)	10 (14)	1 (1)	10 (14)	3 (4)	10 (14)	3 (4)	7 (10)	5 (7)	3 (4)
	R (%)	22 (30)	28 (39)	63 (87)	53 (74)	24 (33)	21 (29)	69 (96)	49 (68)	59 (82)	48 (67)	61 (85)	23 (32)	38 (53)	61 (85)
P. aeruginosa	S (%)	31 (86)	23 (64)	6 (16)	3 (8)	25 (69)	23 (64)	4 (11)	6 (16)	4 (11)	5 (14)	15 (42)	24 (67)	34 (95)	11 (30)
	I (%)	1 (3)	5 (14)	2 (6)	-	4 (11)	3 (8)	3 (8)	1 (3)	-	-	2 (6)	4 (11)	-	2 (6)
	R (%)	4 (11)	8 (22)	28 (78)	33 (92	7 (20)	10 (28)	29 (81)	29 (81)	32 (89)	31 (86)	19 (52)	8 (22)	2 (5)	23 (64)

A.baumannii	S (%)	4 (66)	3 (50)	-	-	3 (50)	2 (33)	1 (17)	-	1 (17)	-	1 (17)	2 (33)	2 (33)	2 (33)
	I (%)	1 (17)	1 (17)	1 (17)	-	-	1 (17)	-	1 (17)	-	1 (17)	1 (17)	1 (17)	2 (33)	-
	R (%)	1 (17)	2 (33)	5 (83)	6 (100)	3 (50)	3 (50)	5 (83)	5 (83)	5 (83)	5 (83)	4 (66)	3 (50)	2 (34)	4 (67)
P. mirabilis	S (%)	4 (66)	4 (66)	1 (17)	2 (34)	2 (34)	1 (17)	3 (50)	-	4 (66)	3 (50)	3 (50)	3 (50)	2 (33)	3 (50)
	I (%)	-	-	-	-	-	1 (17)	1 (17)	1 (17)	1 (17)	1 (17)	-	1 (17)	1 (17)	-
	R (%)	2 (34)	2 (34)	5 (83)	4 (66)	4 (66)	4 (66)	2 (33)	5 (83)	1 (17)	2 (33)	3 (50)	2 (33)	3 (50)	3 (50)
S. marcescens	S (%)	2 (50)	1 (25)	3 (75)	3 (75)	2 (50)	2 (50)	3 (75)	1 (25)	3 (75)	2 (50)	2 (50)	3 (75)	3 (75)	3 (75)
	I (%)	1 (25)	1 (25)	-	-	1 (25)	-	-	-	-	2 (50)	1 (25)	-	-	-
	R (%)	1 (25)	2 (50)	1 (25)	1 (25)	1 (25)	2 (50)	1 (25)	3 (75)	1 (25)	-	1 (25)	1 (25)	1 (25)	1 (25)
Total	S (%)	142 (71)	99 (49)	26 (13)	28 (14)	93 (46)	108 (54)	23 (11)	46 (23)	34 (17)	33 (16)	36 (18)	107 (53)	117 (56)	43 (22)
	I (%)	20 (10)	26 (13)	8 (4)	9 (5)	33 (16)	24 (12)	10 (5)	29 (14)	8 (4)	21 (11)	12 (6)	21 (11)	21 (11)	9 (4)
	R (%)	38 (19)	75 (38)	166 (83)	163 (81)	74 (41)	68 (34)	167 (84)	125 (63)	158 (79)	146 (73)	152 (76)	72 (36)	62 (31)	148 (74)

R: resistant; S: Sensitive; %: percentage; Ptn: Pattern; IPM: imipenem; MEM: meropenem; AML: Amoxicillin; AMP: Ampicillin; CIP: ciprofloxacin; LEV: levofloxacin; CRO: Ceftriaxone; FOX: cefoxitin; CAZ: ceftazidime; CTX: cefotaxime; FEP: cefepime; CN: Gentamicin; AK: Amikacin; CXT: Sulfamethoxazole trimethoprim

Phenotypic Detection of ESBL-producers

Double Disc Synergy Test performed for all the resistant isolates against third-generation cephalosporins including ceftriaxone, ceftazidime, and cefotaxime. *E. coli* 59% (n = 45), *K. pneumoniae* 45% (n = 33), *P. aeruginosa* 50% (n = 18) and *A. baumannii* 33% (n = 2) were found as ESBL producers while 31(41%) *E. coli*, 39(55%) *K. pneumoniae*, 18(50%) *P. aeruginosa*, 4(67%) *A. baumannii*, 6(100%) *P. mirabilis* and 6(100%) *S. marcescens* not determined by the Double Disc diffusion test. Table 5. Confirmed ESBLs

Gram-negative organisms by Double disc synergy test were collected from different sites of hospitals shown in Table 6.

Table 5. ESBLs confirmation of bacterial isolates from Private and Public hospitals of District Jhang through Double Disk Diffusion Test (DDDT)

Antibiotics	E. coli (%)	K. pneumoniae (%)	P. aeruginosa (%)	A.baumannii (%)	P. mirabilis (%)	S. marcescens (%)
Cefotaxime /+Clavulanic acid (30 µg/ 10 µg)	42 (55.2)	33 (45.8)	18 (50.0)	2 (33.3)	-	-
Ceftazidime /+Clavulanic acid (30 µg/ 10 µg)	45 (59.2)	25 (34.7)	13 (36.1)	2 (33.3)	-	-
Ceftriaxone /+Clavulanic acid (30 µg/ 10 µg)	40 (52.6)	29 (40.2)	12 (33.3)	2 (33.3)	-	-
Total (%)	**45 (59.2)**	**33 (45.8)**	**18 (50.0)**	**2 (33.3)**	**-**	**-**

Table.6. Association of ESBLs production with sort of specimens

Specimen	E. coli (n=45)	K. pneumoniae (n=33)	P. aeruginosa (n=18)	A. baumannii (n=2)	Total (n=98)
Door Handles	6	5	2	1	14
Surgical Instruments	6	2	1	-	9
Sink	2	3	2	-	7
Patient Beds	5	3	2	-	10
Infusion Set	4	3	2	-	9
Mounting Sleeves	3	1	1	-	5
Drugs Trolley	4	5	1	-	10
Suction Set	1	2	-	-	3
Floor	3	1	-	-	4
Nebulizer	1	-	-	-	1
E.C.G Sensor	1	-	-	-	1
Operation Theatre	3	-	-	-	3
OPD	2	4	2	1	9
Surgical Ward	2	2	2	-	6
Emergency Ward	2	2	3	-	7

Molecular Identification of ESBLs Producing Gram Negative Bacteria

ESBL-producing isolates (n = 98) as identified through the Combination Disc Test further processed for genes encoding by PCR. 46 (47%) isolates confirmed positive for ESBL encoding genes. Among these total positive ESBL genes, the *bla*-CTX-M gene is predominantly present in 26 (56.52%) strains followed by *bla*-TEM in 13(28.26%) strains and *bla*-SHV in 5 (10.86%) strains. Only two genes bla-CTX-M/*bla*-TEM were present in 1 (2.17%) strain. Moreover, three of these genes *bla*-CTX-M/*bla*-TEM/*bla*-SHV combination found only in 1 (2.17%) strain as shown in Table 7.

Table7. **Prevalence of Extended Spectrum β-*Lactamase (ESBLs)* encoding genes amongst Isolates**

Isolate	E. coli (n=22)	K. pneumoniae (n=16)	P. aeruginosa (n=8)	Total Prevalence (n=46) 47.0%
bla_{TEM}	6 (27.3%)	5 (31.25%)	2 (25.0%)	13 (28.26%)
bla_{SHV}	2 (9.0%)	1 (6.25%)	2 (25.0%)	5 (10.86%)
bla_{CTX-M}	13 (59.0%)	9 (56.25%)	4 (50.0%)	26 (56.52%)
bla_{TEM}/ bla_{CTX-M}	1 (4.54%)	0 (0.0%)	0 (0.0%)	1 (2.17%)
bla_{TEM}/bla_{SHV}/bla_{CTX-M}	0 (0.0%)	1 (6.25%)	0 (0.0%)	1 (2.17%)

Among 22 ESBLs positive strains of *E. col,* only single gene *bla*-CTX-M was predominantly present in 13 (59%) strains, *bla*-TEM was present in 6 (27.3%) strains and single gene *bla*-SHV was present in 2 (9%) strains. Two genes *bla*-CTX-M and *bla*-TEM were present in 1 (4.54%) strain. Similarly, in the case of *K. pneumoniae* single gene *bla*-CTX-M was predominantly present in 9 (56.25%) strains, *bla*-TEM was present in 5 (31.25%) strains and single gene *bla*-SHV was present in 1 (6.25%) strain. All of these three genes *bla*-CTX-M*, bla*-TEM, and *bla*-SHV present in 1 (6.25%) among *K. pneumoniae* strains (Table 8.)

Table 8. Prevalence of Extended Spectrum β-*Lactamase (ESBL)* encoding genes *bla*-CTX-M, *bla*-TEM, and *bla*-SHV in *Escherichia coli*

Sample. No.	TEM	SHV	CTX-M
1	-	-	+
2	+	-	-
3	-	-	+
4	-	-	+
5	-	+	-
6	+	-	-
7	-	-	+
8	+	-	+
9	-	-	+
10	-	-	+
11	-	-	+
12	+	-	-

13	-	-	+
14	-	-	+
15	+	-	-
16	+	-	-
17	-	-	+
18	-	-	+
19	-	+	-
20	-	-	+
21	-	-	+
22	+	-	-

In case of *K. pneumoniae* single gene *bla*-CTX-M was predominantly present in 9 (56.25%) strains, *bla*-TEM was present in 5 (31.25%) strains and single gene *bla*-SHV was present in 1 (6.25%) strain. All of these three genes *bla*-TEM, *bla*-CTX-M, and *bla*-SHV were present in 1 (6.25%) among *K. pneumoniae* strains Table 9.

Table 9. Prevalence of Extended Spectrum β-*Lactamase (ESBL)* encoding genes *bla*-TEM, *bla*-SHV and *bla*-CTX-M in *Klebsiella pneumoniae*

Sample. No.	TEM	SHV	CTX-M
1	-	+	-
2	+	-	-
3	-	-	+
4	+	+	+
5	-	-	+
6	+	-	-
7	+	-	-
8	-	-	+
9	-	-	+
10	-	-	+
11	+	-	-
12	-	-	+
13	+	-	-
14	-	-	+
15	-	-	+
16	-	-	+

Among 8 ESBL positive encoding genes strains of *P. aeruginosa,* single gene *bla*-CTX-M was predominantly 4 (50%) strains, *bla*-SHV 2(25%) and single gene *bla*-TEM was present in 2 (25%) strains Table 10.

Table.10. Prevalence of Extended Spectrum β-*Lactamase (ESBL)* encoding genes *bla*-TEM, *bla*-SHV and *bla*-CTX-M in *Pseudomonas aeruginosa*

Sample. No.	TEM	SHV	CTX-M
1	-	-	+
2	-	+	-
3	-	-	+
4	+	-	-
5	-	+	-
6	+	-	-
7	-	-	+
8	-	-	+

Discussion

The ESBLs producing bacteria prevalence in hospitals positions is a severe challenge. This problem affects both developing and developed regions of the world. Many harmful bacteria strains are progressively expressing these enzymes, which has the potential to spread. The presence of ESBL impairs the efficacy of wide-spectrum antibiotics, posing considerable treatment challenges and affecting patient outcomes. Clinical microbiology laboratories have diagnostic concerns as ESBLs continue to appear.

This research aimed to investigate the ESBLs producing gram-negative isolates susceptibility to certain antimicrobial drugs and Molecular identification of ESBLs producing genes. The Enterobacteriaceae family, in particular *E. coli, K. pneumoniae, P. aeruginosa* and *A. baumannii* primarily produces ESBLs. Reports from all around the world shown that different countries have different proportion of ESBL producing isolates [27]

In current research, ESBLs discovered to be 49%. On the other hand, the rate of ESBL generation differs 17% to 70% amongst research [28-30]. In this research, 59% Escherichia coli, 46% Klebsiella pneumoniae, 50% Pseudomonas aeruginosa and 33% Acinetobacter spp. present. According to an Indian study, higher rates 66.7% of ESBL-producers among the total Gram-negative strains, 81% *Escherichia spp,* 74% *Pneumoniae spp* and 14% *Pseudomonas species* were ESBL producer [31].

The highest bacterial contaminated samples obtained from Door handles of different wards isolated *Escherichia coli* 43.75% and *Klebsiella pneumoniae* 37.5% followed by Surgical Instruments, Sink and beds in this study while other research showed that the suction tube was the most contaminated with bacterial pathogens including *Pseudomonas aeruginosa* and *Proteus mirabilis* [32]. Among various Gram-Negative isolated bacteria, ESBL production was highest in Escherichia coli, but in some cases, Klebsiella spp was common source of ESBL producer [26]. In accordance to other research, ESBL production is less in Klebsiella spp. and Pseudomonas spp. as compared to E. coli which is in current research [31,33-34].

The most common antibacterial classes to treat infections caused by these bacteria include the beta-lactams, aminoglycosides, carbapenems, fluoroquinolones and trimethoprim sulfamethoxazole. In present study, isolates were sensitive to imipenem 71%, amikacin 69%, levofloxacin 66%, and gentamicin 64% while high resistance recorded to most of the antibiotics including ceftriaxone 84%, amoxicillin 83%, ampicillin 81%, ceftazidime 79%, cefepime 76%, sulfamethoxazole trimethoprim 74%, cefotaxime 73% and cefoxitin 63%. Whereas according to another study, the proportion of the Gram negative bacterial resistant to cephalothin 77.7%, cefotaxime 86.1%, and ceftazidime 91.7% respectively which are similar to our study [35]. Antimicrobial resistance in Gram-negative bacteria has increased

dramatically in the last two decades over the world [36], which has remained connected to an increase in infection-related mortality, morbidity, and hospital expenses. Similarly, majority of Enterobacter organisms are either extremely resistant to a wide range of antimicrobial drugs or might develop resistance during treatment, making the selection of effective antimicrobial medicines difficult [37]. Main proportion of the gram-negative bacteria resistance to 3rd generation cephalosporins including ceftazidime, ceftriaxone and cefotaxime further subjected to ESBL producer through phenotypic detection of isolates by double disc diffusion test. In current research, maximum prevalence of ESBLs positive strains detected in E.coli 59% followed by K. pneumoniae 45%, P. aeruginosa 50% and A. baumannii 33% determined as ESBL producers. According to a study in India, ESBL production in Acinetobacter baumannii 72%, Proteus mirabilis 61% which quite high as compared to present study [38]. On contrast, among isolated various gram-negative bacteria ESBL production 67.04% observed in Klebsiella spp. after that 56.92% in Escherichia coli, 46% Proteus spp., 41.89% Pseudomonas spp. and 11.11% *Acinetobacter spp*., which is quite different from present study [26].

However, only molecular detection methods allow for conclusive identification. Here are so many types of ESBLs like TEM, SHV, CTX, OXA, AmpC etc. However, the bulk of ESBLs are variants of CTX-M, TEM and SHV enzymes, which are often detected. TEM, SHV, and CTX-M genes amplified using a set of forward and reverse primers. In present study, among the total ESBL gene positive isolates *bla*-CTX-M gene predominant present 56.52% strains followed by *bla*-TEM in 28.26% strains and *bla*-SHV in 10.86% strains as detected by the PCR. Similar finding from several regions around the world as previously investigated [39]. ESBL-producers are very common (87.5%), although this is due to research on metallo-lactamase producers, which are extremely resistant organisms [40]. A report from Lahore (Pakistan) showed that *bla*-CTX-M genes prominent 76% followed by *bla*-TEM 28% and *bla*-SHV 21% [41]. In current research, *bla*-CTX-M was present in 56.52% but Several research from throughout the world have revealed varied prevalence rates of blaCTX-M gene including 98.8% in China, 84.7% in Chile and 13.6% in Tanzania [42-44]. The mainstream of Malaysian ESBL-positive isolates had 88% *bla*-TEM, it is extremely high as compared to our research, however they testified 20% occurrence of *bla*-CTX-M and 8% of SHV that is low as compared to current study [45]. According to a study in India, the majority of strains (57.3%) have two or more ESBL genes. [45], which is quite high as compare to this study, two genes bla-CTX-M/*bla*-TEM was present only in 2.17% strains. Moreover, three of these genes *bla*-CTX-M/*bla*-TEM/*bla*-SHV combination was present in only 2.17% of the isolates.

With the passage time, different ESBL-producing strains become resistant to different antibiotics, it is critical to discover these strains early in microbiology laboratories. Treatment with antibiotics played a critical role in the management of bacterial illness. However, drug resistance has evolved because of ESBL enzymes degrading all beta lactam antimicrobial drugs, causing bacteria to become resistant. Furthermore, plasmids with a high plasmid burden were discovered to take part in gene transfer and to carry extra antibiotic resistance genes in addition to -lactam antibiotics.

Conclusion

The current research emphasizes the significant incidence of ESBLs producing bacterial isolates in the region of Jhang, Punjab, Pakistan. Most of the isolates were resistant to certain antimicrobial drugs and maintained their sensitivity to imipenem. In light of the increasing medication resistance, the use of ESBL testing in conjunction with traditional antibiotics would be advantageous in all circumstances and would aid in the patient's proper therapy and avoid additional expansion of bacterial drug resistance. Molecular typing of isolates would regulate which types of ESBLs are present. For epidemiological analysis of antimicrobial resistance, molecular detection, and identification of ESBLs would be essential. These enzymes can be plasmid mediated or chromosomal that could support in propagation of antimicrobial resistance in hospitals. Therefore, ESBLs producing isolates must be identified rapidly to facilitate suitable antimicrobial treatment and infection control processes can be applied.

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