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Research Article

Phenotypic and Genotypic Characterization of ESBL Bacteria in the Human Population

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ABSTRACT

Growing occurrence of antimicrobial resistance (AMR) in humans has become an intense global concern. Dissemination of extended-spectrum beta- lactamase (ESBL) producing *Escherichia coli* across the geographic boundaries is causing a grave challenge to the efficacy of antimicrobial agents. The purpose of this work is to demonstrate the mechanism of antibiotic resistance patterns prevailing in human interface and the need to employ one health approach to counter these alarming trends. Our objective was to inspect the MDR (Multi Drug Resistant) Enterobacter isolates for specific ESBL producing genes (CTX-M, SHV, TEM and OXA) and elicit the resistance profiling of Enterobacteriaceae family using conventional PCR. The study also intended to establish a One Health link in the dissemination and incidence patterns of ESBL bacteria. Bacterial strains were isolated by employing Gene JET Gel genomic kit. After isolation of bacterial DNA and gel electrophoresis clear DNA bands in the 1% agarose gel were obtained. Out of 100 isolates, 31 (31%) isolates were ESBL producers showing a greater degree of resistance to antibiotics. Its prevalence is not common in humans. Highest level of resistance was found in Amoxicillin followed by Ceftriaxone. Elements of resistance were also found in TZP, NIT and CTX in varying degrees. The resistance pattern in the samples taken from human were almost the same with minute differences. This indicates that a common resistance mechanism is operating across these different sources, while also highlighting multiple pathways through which humans are exposed to resistant bacteria and their associated resistance genes. Future work in this area will need enhanced statistical capabilities and multiple samples from humans. Techniques of molecular biology like meta-genomic analysis of entire DNA, and bioinformatics analytical tools have the potential to replace current techniques.

Keywords: Antimicrobial resistance, ESBL, Multidrug resistance, Enterobacteriaceae, Human interface.

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INTRODUCTION

Discovery of antibiotics is one of the key milestones of history enabling human race to effectively fight the deadly diseases caused by pathogens. Addition of antibiotics in the arsenal has helped to save millions of human lives. However, indiscriminate use of antibiotics has its own adverse effects (Carrique-mas, 2017). This emerging and evolving phenomenon is putting the future of humanity at risk (Ojer-usoz et al., 2017). Irresponsible use of antibiotics can have dangerous consequences, and it can potentially impact the health of our future generations as well. Antimicrobial resistance, commonly known as AMR is a product of twentieth century and its impact is growing at a rapid pace (Science et al., 2016). AMR is the capacity of microorganisms to evolve against the administered drugs, enabling them to counter their action, consequently making those drugs Ineffective (Karaiskos et al., 2019).

Antimicrobial resistance (AMR) is regarded as a worldwide health concern (Carrique-mas, 2017). Recent studies indicate that there are also human health concerns about the presence of antimicrobial residues in meat (R. M & F, 2008) and eggs (V et al., 2011). The cumulative approach to deal with the growing threat of AMR is termed as One Health Approach. The main theme is that human as the antibiotics are mostly similar to those administrated for human treatment. The normal geographical borders and human segregation are not recognized by AMR. One Health is an effective mechanism to deal with an emerging issue like AMR. This approach is unique as it successfully studies and inter link the sectors of human.

The concept of AMR has gained global acceptance due to multiple factors. The main considerations are emergence of multiple drug resistant bacteria, and lack of new antimicrobials. It is a well-established fact that Extended- spectrum beta-lactamase (ESBL) producing Escherichia coli and other zoonotic pathogens can be transmitted from one set of hosts to another such as to humans and vice versa. It has been observed that there is an increased resistance to medically important antibiotics such as cephalosporin, carbapenem, aminoglycosides, and fluoroquinolones among bacteria of zoonotic importance (Bitrus et al., 2019). A better understanding of main factors will assist in reducing the resistance level, thus increasing the curative efficiency of antimicrobial agents for the treatment of both human diseases resulting from chronic infections. Dissemination of extended-spectrum beta-lactamase (ESBL)-producing Escherichia coli across the geographic boundaries is causing a grave challenge to the efficacy of antimicrobial agents, particularly the third- and fourth-generation cephalosporins (Cantón et al., 2008). The mode of action of ESBLs enzymes is that they inactivate beta-lactams antibiotics mostly penicillin and third generation cephalosporins. The phenomena occur through hydrolysis of their beta-lactam ring. These enzymes are prevalent in the normal gut flora of Enterobacteriaceae. It has also been witnessed that last resort antibiotics such as colistin and carbapenems are being prescribed as first line of defense leading to the emergence of E. coli strain resistant to carbapenem and colistin. This in turn creates a momentous health issue in developing countries. Rapid dissemination of AMR genes in human is a direct result of lack of good sanitation in these countries (Maria et al., 2019).

Objectives

Identification of MDR, ESBL bacteria using AST (Disk Diffusion Method). Qualitative screening of ESBL Enterobacter species (*E. coli, Klebsiella pneumoniae, Salmonella arizonae, Pseudomonas aeruginosa, Proteus vulgaris*) using Double Disk Synergy Test. Molecular identification of β-lactamase resistant genes in ESBL Enterobacter species (*E. coli, Klebsiella, Salmonella*) using Multiplex PCR.

MATERIALS AND METHODS

This research work was carried out in National Veterinary Laboratories, Islamabad, over a period of six months. The samples were collected from Human population in Islamabad Capital Territory. Samples (stool, urine, blood) were collected aseptically and transported to NVL Islamabad at refrigerated temperature. All samples were collected under sterile conditions.

Sampling

Venesection (Blood) Samples

The procedure was done after making sure the patient is comfortable first. A 10 ml syringe was inserted directly into vein. Blood was drawn out and collected for sampling. After sampling, it was transported to National Veterinary Labs Islamabad (Burgrass, 2022).

Urine Samples

Midstream urine samples were collected in sterile containers and placed in plastic bags. Each container was then removed from the bag and properly labeled, with the date and time of collection clearly recorded. The subject's name and date of birth were verified in the subject's presence prior to submission of the sample to the laboratory. A total of 10 urine specimens were taken and sent to National Veterinary Laboratories, Islamabad. Samples that were not immediately processed were kept under refrigerated conditions at 2-8 degrees C until analysis (Poljak et al., 2023).

Stool Samples

Stool samples were collected from different participants in sterile 4 ounce specimens collection containers that were placed in biohazard specimen bags, with time of sample collection duly noted. Each stool sample was aliquoted in four different 1.8 mL dry cryovials using sterile tongue depressor or wooden applicator following the criteria that each aliquot contained at least 0.5 mL of sample. The cryovials were properly labeled as to date and time of collection. After aliquoting, the samples were kept in a freezer at -70 degrees or lower until the samples were transported. A total of 10

stool samples were taken and immediately transported to the National Veterinary Laboratories, Islamabad (Gemmell et al., 2024).

Isolation and Identification of Bacterial Pathogens

Samples were processed by standard micro biological methods to isolate and identify organisms belonging to bacterial family Enterobacteriaceae, namely ESBL producing *Escherichia coli*, *Klebsiella* spp., and *Salmonella* spp. Multidrug resistant (MDR) strains of *Salmonella*, *E. coli*, *Klebsiella*, *Pseudomonas*, and *Proteus* were also targeted.

Preparation of Culture Media

ESBL-Selective MacConkey Agar (MAC-CEF)

MacConkey agar was prepared using 50 g powder dissolved in 1 L sterile water and subjected to boiling and autoclaving at 121 ^{0}C for 15 min. A cefotaxime (CTX) stock solution (4 mg/mL) was prepared and added to the 50deg C cooled medium. The medium was thoroughly mixed and poured into Petri plates that were sterile (Picton-Barlow et al., 2025).

Buffered Peptone Water (BPW)

Buffered peptone water was prepared by dissolving 20g powder in 1L distilled water, boiled in a test tube and autoclaved at 121 degree centigrade for 15min (Wages et al., 2022).

Mueller–Hinton Agar (MHA)

Mueller- Hinton agar was prepared by dissolving 38 g powder in 1 litre distilled water, boiled, autoclaved, cooled and poured into sterile Petri plates for antimicrobial susceptibility test (Chelaru, et al., 2025).

Physiological Saline Solution

An 8.5 g sodium chloride was dissolved in 1 liter distilled water for preparing saline solution. The pH was adjusted to pH 7.0 and autoclaved and dispensed into sterile tubes (Abubakar et al., 2025).

ESBL-Selective SS Agar (SS-CEF / DCA)

Deoxycholate citrate agar was made and supplemented with cefotaxime after cooling. The medium was loaded in sterile petri plates for the selective isolation of ESBL-producing organisms (Diarra et al., 2024).

Salmonella–Shigella (SS) Agar and XLD Agar

SS agar and XLD agar were prepared with powder of both, 50 g powder in 1 liter distilled water boil and cool and poured into sterile Petri plates (Neyaz et al., 2024).

Biochemical Identification

Gram Staining

Bacterial smears were prepared, heat-fixed and stained with crystal violet, iodine, ethanol and safranin. Slides were observed under the microscope by oil immersion (100x) to find the Gram reaction (Paray et al., 2023).

Indole Test

Tryptone water was inoculated with the overnight bacterial culture and placed in 37 degC for 24 hours. Indole reagent was added and the appearance of red ring showed the occurrence of positive reaction (Niu et al., 2023).

Methyl Red (MR) Test

Then MR-VP broth was inoculated and incubated at 37 degC for 24 hours. The addition of methyl red indicator in red color indicated the positive result (Bouzid et al., 2023).

Voges–Proskauer (VP) Test

Following incubation, VP reagents were added to the broth culture. Development of pink to red color within 5 minutes confirmed a positive reaction (Maisya et al., 2023).

Citrate Utilization Test

Simmons citrate agar slants were inoculated and incubated at 37 °C for 24 hours. A color change from green to blue indicated citrate utilization (Roy et al., 2023).

Antimicrobial Susceptibility Testing (AST)

Antimicrobial susceptibility testing was performed using the Kirby–Bauer disk diffusion method on Mueller–Hinton agar. Standardized bacterial inoculum (0.5 McFarland) was prepared and evenly spread on agar plates. Antibiotic discs were

applied, and plates were incubated at 37 °C for 18 hours. Zones of inhibition were measured and interpreted according to CLSI or EUCAST guidelines (Gajik et al., 2022).

Phenotypic Confirmation of ESBL Production

Phenotypic confirmation of ESBL production was carried out using two methods: the Combination Disk Test (CDT) and the Double Disk Synergy Test (DDST).

In the Combination Disk Test, cephalosporin disks (cefotaxime or ceftazidime) and the same antibiotics combined with clavulanic acid were placed on Mueller–Hinton agar plates inoculated with the test organism. After incubation, an increase of 5 mm or more in the inhibition zone around the antibiotic–clavulanic acid disk compared to the antibiotic alone was considered positive for ESBL production (Harris, et al., 2023).

In the Double Disk Synergy Test, cephalosporin disks were placed at a distance of 20 mm (center to center) from an amoxicillin–clavulanic acid disk on Mueller–Hinton agar. After incubation, enhancement of the inhibition zone toward the clavulanic acid disk indicated ESBL production (Yilmaz et al., 2022).

Genotypic Characterization of ESBL Genes

DNA Extraction

Genomic DNA was extracted from phenotypically confirmed ESBL-producing isolates using the GeneJET Genomic DNA Purification Kit following the manufacturer's instructions. Briefly, bacterial colonies were suspended in normal saline and centrifuged to obtain a pellet. The pellet was treated with digestion solution and proteinase K, followed by incubation at 56 °C. RNase treatment and lysis solution were applied to remove RNA and lyse the cells. Ethanol was added and the mixture was transferred to a purification column. DNA concentration was eluted and obtained with elution buffer; it was then immediately used or stored at -20 per ^{0}C for subsequent analysis (Schenk et al., 2023).

Polymerase Chain Reaction (PCR)

Conventional PCR was used for the detection of ESBL genes which include blaCTX-M, blaSHV, blaTEM, blaOXA by gene specific primers. PCR reaction solution was composed of deionized water, Green PCR Master Mix, MgCl2, dNTPs, primer for forward and reverse, Taq DNA polymerase, and template DNA with a final volume of 20 ul. PCR amplification was performed in a thermal cycler under standard conditions consisting of initial denaturation and 30-35 cycles of denaturation, annealing, and extension, and a final extension. The annealing temperature was dependent on the primer set used (Mareso et al., 2023).

Agarose Gel Electrophoresis

PCR products were analyzed using agarose gel electrophoresis. A 1.5% agarose gel containing ethidium bromide was prepared in 1× TAE buffer. PCR amplicons along with a 100 bp DNA ladder were loaded into the gel wells. Electrophoresis was performed at appropriate voltage, and the gel was visualized under ultraviolet light using a gel documentation system. The presence of specific ESBL genes was confirmed by comparing band sizes with the expected product sizes (Arakawa et al., 2022).

RESULTS

Sampling

25 human (Blood, Urine, Stool) and samples were collected from Islamabad Capital Territory. The details of biochemical; tests are given in table 1.

Table 1. Morphological and Biochemical Characterization of bacteria.

Microbe	Colony Morphology	Gram Staining	Indole	Methyl Red	Vogeus prausker	Citrate	TSI	Urease	Catalase
Salmonella	transparent colonies on SS agar with black centers	gram -	-	+	-	-	+	-	-
ESBL *Salmonella*	transparent colonies on SS agar with black	gram -	-	+	-	-	+	-	-

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	centers								
E. coli	Pink colonies	gram -	+	+	-	-	+	-	-
ESBL *E. coli*	Pink colonies	gram -	+	+	-	-	+	-	-
Pseudomonas	blue green and yellow green colonies on selective cetrimide agar	gram -	-	-	-	+	Y/Y Ga s +	-	+
Klebsiella	large, shiny, dark pink in colour on MacConkey agar	gram -	-	-	+	+	A/A	+	+
Proteus	Smooth, colorless colonies	gram -	-	+	-	+	No gas -	+	+

Double Disk Diffusion Test

Antibiotic susceptibility testing of 25 human clinical samples was performed using the Kirby–Bauer disc diffusion method. From these samples, Gram-negative bacterial isolates including Escherichia coli, Salmonella, Klebsiella, Pseudomonas, and Proteus were recovered. Extended-spectrum β-lactamase (ESBL) production was detected phenotypically by the double disk synergy test, with ESBL-producing strains identified among E. coli and Klebsiella isolates from human samples.

Table 2. Table: Distribution of Bacterial Isolates and ESBL Producers from Human Samples.

Sample Type	Total Samples	*E. coli*	ESBL *E. coli*	*Salmonella*	ESBL *Salmonella*	*Klebsiella*	ESBL *Klebsiella*	*Pseudomonas*	*Proteus*
Blood	6	–	–	–	–	1	–	5	–
Stool	13	5	2	5	–	–	–	1	–
Urine	6	–	3	–	–	–	3	–	–
Total	25	5	5	5	0	1	3	6	0

A total of 25 human clinical samples were analyzed, from which *Escherichia coli*, *Klebsiella*, *Pseudomonas*, and *Proteus* were isolated. ESBL production was detected phenotypically in *E. coli* and *Klebsiella* isolates. Most Enterobacteriaceae were resistant to amoxicillin–clavulanic acid, piperacillin–tazobactam, ciprofloxacin, and gentamicin, while all ESBL-producing isolates were sensitive to imipenem. High resistance was observed against ceftriaxone, and the lowest resistance was recorded against imipenem. PCR screening revealed the presence of SHV and CTX-M genes in human ESBL isolates, whereas TEM and OXA genes were not detected.

Resistance Patterns

A total of 8 ESBL isolates were found from Humans.

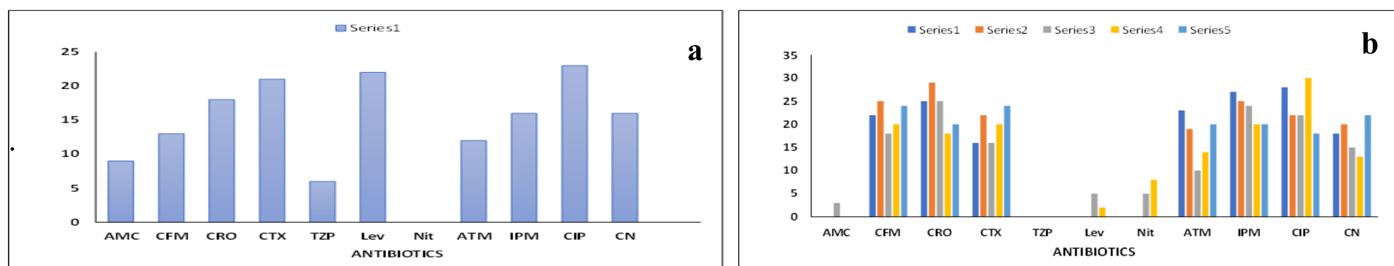

Figure 1. Resistance patterns of Blood (a) *Klebsiella* (b) *Pseudomonas* against different drugs.

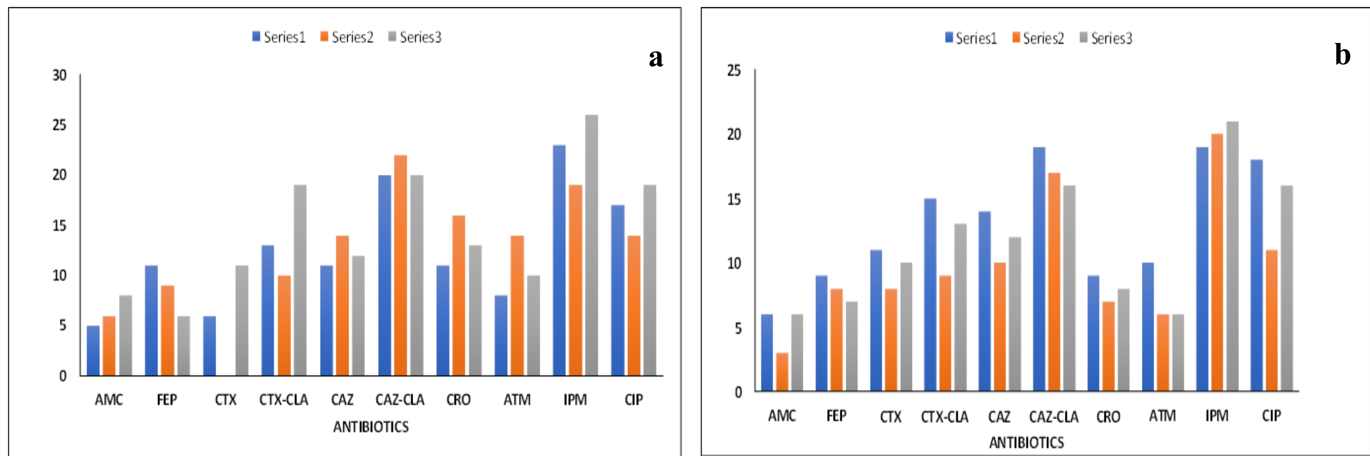

Figure 2. Resistance patterns of Urine ESBL (a) *E. coli* (b) *Klebsiella* against different drugs.

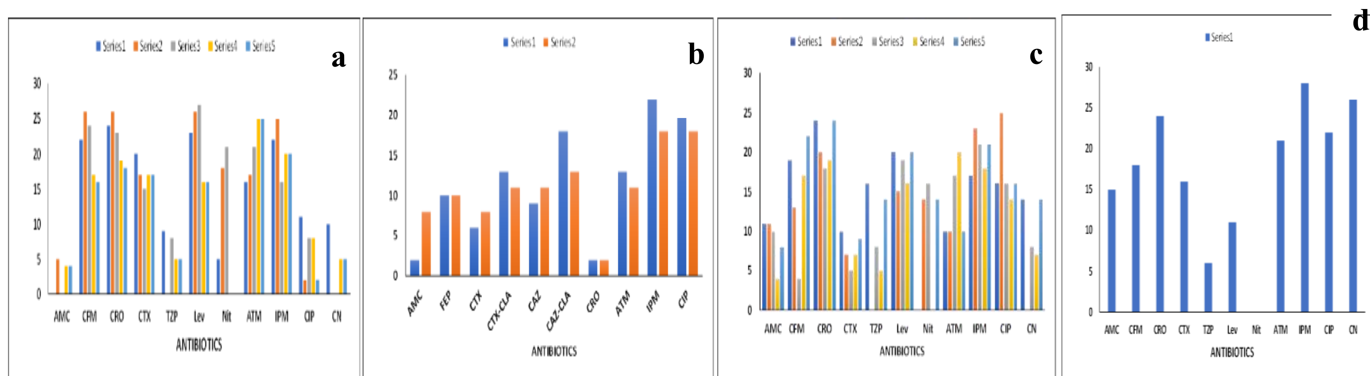

Figure 3. Resistance patterns of Human Stool (a) *E.coli* (b) ESBL *E.coli* (c) *Salmonella* (d) *Pseudomonas* against different drugs.

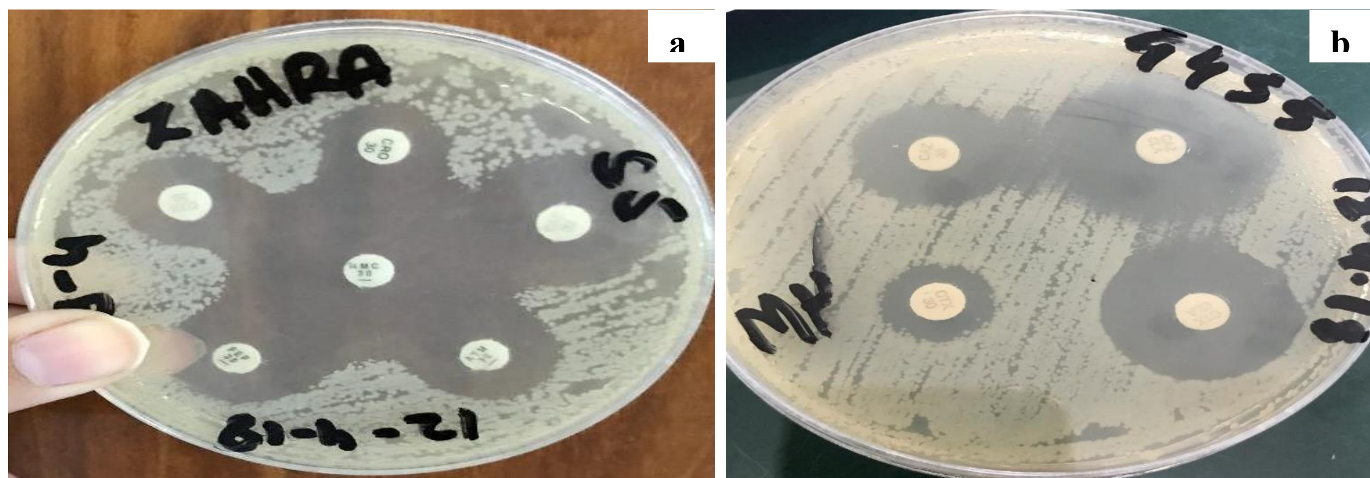

Figure 4. (a) Kirby Bauer Disk Diffusion Test (b) Double Disk Synergy test for the Confirmation of ESBL.

Genotypic Results

CTX-M and SHV was found in various Clinical samples collected from Human Urine, Stool and Blood. However TEM and OXA were absent.

Table 3. Details of ESBL genes found in Human.

Sample #	CTX-M	SHV	TEM	OXA
1.	+			
2.	+			
3.	+			
4.	+	+		
5.	+			
6.	+			
7.	+			
8.	+			

DISCUSSION

Enterobacter bacteria are an important group of organisms due to their multidrug resistance, with extended-spectrum beta-lactamases (ESBLs) being the most significant mechanism of resistance. ESBLs are derived from broad-spectrum beta-lactamases such as TEM-1, TEM-2, and SHV-1 by a limited set of mutations and exist in multiple forms, including TEM, SHV, OXA, CTX-M, and AmpC (Sharma). These enzymes are present in *E. coli* and *K. pneumoniae* and confer resistance against multiple antibiotics. In this study, human isolates of *E. coli*, *K. pneumoniae*, *Salmonella*, *Pseudomonas aeruginosa*, and *Proteus* were analyzed, with *E. coli* and *Klebsiella* identified as the most prevalent ESBL-producing organisms.

The phenotypic existence of ESBLs in isolates became verified with precise PCR conditions and gene specific primers. CTX-M and SHV genes were the most commonly found whereas TEM and OXA were not found among human samples. This high prevalence of CTX-M is in fact in accordance with the results of Razib et al. (2020), which reported CTX-M as the most prevalent ESBL gene. High resistance to third- and fourth-generation cephalosporins, amoxicillin, ceftriaxone and aztreonam was observed in human isolates while sensitivity to imipenem was observed in all ESBL-producing isolates. These results are similar to the findings obtained for ESBL producing Enterobacteriaceae which exhibit a reduced susceptibility to most of the antibiotics except the carbapenems and amikacin (Subramanya et al, 2021).

Among the uropathogens, E. coli was the most common microorganism, which is in agreement with reports by Tanko Nuhu et al. (Manikandan et al., 2011; Abubakar, 2009; Akinyemi et al., 2007). The virulence factors such as hemolysin formation and fimbriae are responsible for its dominance in urinary tract infections (Abubakar, 2009; Al-Jebouri and Mdish, 2013). K. pneumoniae was the second most common uropathogen, with 7 (7 out of 15) isolates all of which were multidrug resistant as well as 8 ESBL-positive. These findings are in line with earlier studies on the high MDR prevalence in *K. pneumoniae* (Teklu et al., 2019).

Opportunistic pathogen, *Pseudomonas aeruginosa* was isolated from human blood and stool specimens were positive for carbapenemase production as revealed in double disk synergy tests. These isolates showed high level of resistance to nitrofurantoin and tazobactam, intermediate level of resistance to cefixime and amoxicillin and susceptibility to aztreonam, imipenem, ciprofloxacin and gentamicin.

The study confirms that the resistance to antibiotics is prevalent in human clinical isolates, reflecting the impact of excessive antibiotic consumption and lack of proper infection control activities in developing countries such as Pakistan (Laxminarayan & Bhutta, 2016). The presence of ESBL genes CTX-M and SHV in the human isolates reflects the genetic basis of resistance, and reveals the need for One Health strategies to track and curb the spread of resistant pathogens (Teklu et al., 2019).

CONCLUSIONS

ESBL and carbapenemase producing bacterial isolates are emerging at an accelerated rate in human clinical settings in our nation. The intrinsic and acquired resistance mechanisms of ESBL bacteria are limited in treatment alternatives during medical emergencies. This realization has resulted in the heightened urgency by researchers and policy makers that has led to greater resources being allocated to the study of multidrug-resistant (MDR) phenomena. The main challenge is to ensure the safe administration of antimicrobial agents to reduce the proliferation of resistant bacteria in human populations.

Numerous medically important bacterial species are resistant to widely used antibiotics, resulting in the emergence of highly resistant “super bacteria” with pan-resistance to antimicrobial therapies. β-Lactamase-producing Gram-negative

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bacilli are now widely reported in clinically significant bacteria, including Enterobacteriaceae, *Pseudomonas* spp., and *Acinetobacter* spp. More than 800 β-lactamases have been recognized in Gram-negative bacilli, with approximately 120 types established in *E. coli*. Members of the Enterobacter family have developed a wide range of antibiotic-resistance genetic variants, often co-existing with virulence genes. In human isolates, there was a high prevalence of the beta-lactamase enzymes blaCTX-M and blaSHV, whereas blaOXA and blaTEM were found in Pseudomonas aeruginosa too.
Establishing mechanisms of resistance and knowing factors that affect their spread is critical. It is essential to have routine screening of ESBL's and carbapenems before treatment with antibiotics. Co-existence of carbapenemase production with ESBLs or virulence genes with clinical isolates is an alarming emerging danger. Rapid detection of ESBLs in humans is required for appropriate and prompt therapeutic treatment and infection control efforts. Moreover, the growing prevalence of antibiotic resistance has highlighted the urgent need for the development of new antimicrobial agents, and the investigations to develop effective b-lactamase inhibitors, in order to restore the efficacy of existing b-lactam antibiotics.

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AUTHOR CONTRIBUTIONS
All the authors contributed equally to this research.

COMPETING OF INTEREST
No conflicts of interest have been disclosed by the authors.

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