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Antibiogram and molecular characterization of multi-drug resistant microorganisms isolated from urinary tract infections

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Abstract: Bacteria are the commonest etiological factor among the microbes that cause UTIs. The most prevalent bacteria identified in the lab are *Escherichia coli*, *Klebsiella pneumonia*, and *Pseudomonas aeruginosa*. Antibiotics are the empiric therapy for such infections but the reoccurrence rate is becoming high owing to the development of resistance due to their irrational and indiscriminate use across the globe. This study was designed on UTI cases of OPD, Medical, Nephrology, Surgical, Main OT, Urology and ICU wards of Allied hospital Faisalabad. 11 antibiotics were used which showed that *E. coli* is sensitive to Amikacin, Gentamicin, Imipenem, Piperacillin tazobactam, and Polymyxin B. *Klebsiella pneumonia* showed sensitivity for Amikacin, Gentamicin, Nitrofurantoin, Imipenem, Polymyxin B, Piperacillin tazobactam and Trimethoprim-sulfamethoxazole. While *Pseudomonas aurignosa* showed resistance to Amikacin, Ciprofloxacin, Gentamicin, Piperacillin tazobactam, Imipenem, and Polymyxin B. *E. coli* exhibited the highest sensitivity for Piperacillin tazobactam, *Klebsiella pneumonia* for Imipenem and *Pseudomonas aurignosa* for Ciprofloxacin. Further, the isolated DNA samples of these microorganisms were confirmed by gel electrophoresis and subjected to molecular characterization by performing trace file and phylogenetic tree analysis.

Keywords: Antibiotics, resistance, characterization.

INTRODUCTION

Urinary tract infections (UTIs) include the commonly occurring diseases that occur by the rise of normal enteric flora into the bladder through the urethra. UTIs incidence is ever increasing with time due to the development of antibiotic resistance among the uropathogens (Kutasy, Coyle *et al*., 2017). It is recorded that China, Thailand, Philippines, and India showed that a greater number of Gram-negative species produced *E. coli* ß- lactamase (Zahar, Poirel *et al*., 2015). 150 million patients suffer from UTI worldwide, sequencing more than 6 billion US $ loss per annum.

The microbial agents causing UTI are bacteria, fungi, and parasite, but mostly UTI is of bacterial origin. Mostly, uropathogens belong to the Enterobacteriaceae family (Roy, Reddi *et al*., 2016). These infections are the most prevalent, affecting people at a community as well as hospital level. The bacteria involved in the UTI have special properties of toxins production, adhesion and siderophores secretion, which enable them to invade the urinary tract and transmit infection among various individuals (Luthje and Brauner, 2014). The rate of ever-increasing UTI is due to the overuse of antibiotics as a treatment therapy. *E. coli*, one of the top bacterial causes of UTI, is a member of the colon's normal flora. *E. coli* is mainly present in the epithelial duct of animals and humans (Tena, Gonzalez-Praetorius *et al*., 2008). The identified *E. coli* strains are more than 700. *E. coli* accounts for 43-73% for infections, *Klebsiella pneumonia* 7-12% and *Pseudomonas aurignosa* 1-5% of infections (Karlowsky, Lagace-Wiens *et al*., 2011). Uropathogenic *E. coli* leads to approximately 90% of UTIs and is the most isolated microbe in UTI patients (Farajnia, Alikhani *et al*., 2009).

According to The Global Prevalence Infection in Urology (GPIU) studies, Urinary tract infection (UTI) incidence ranks the third after gastrointestinal and respiratory diseases. Six million patients in outpatient departments (OPD) and 300,000 in the wards are treated every year for UTI throughout the world (Prakash and Saxena, 2013).

The chromosomes together make up the genome of a living being. This genome is present in the nucleoid of a cell. It encodes for all the vital biochemical reactions essential for living. Furthermore, pathogenic organisms may carry hereditary highlights which are obligatory for destructiveness. Therefore, in this research, the samples of DNA of *E. coli*, *Klebsiella pneumonia*, and *Pseudomonas aurignosa* were sequenced to analyze these microbes' genomes. Analysis and comparison of DNA sequences were performed using different bioinformatics tools, such as trace file analysis and phylogenetic tree analysis.

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MATERIALS AND METHODS

Materials

Requirements of laboratory apparatus

The laboratory instruments which were required for the procedure are as follows: Sterile EDTA tubes, alcohol swabs, gloves, masks, sterile cotton swabs, Petri plates (25ml), Flask (200ml-500ml), Beaker (200ml-500ml), Test tubes (20ml), Glass slides, Sterile syringes, Micropipettes, Sterile tips, Eppendorf tubes, Inoculation platinum wire loop, Bunsen burner, incubator (temp. range 35-37oC), Hot air oven, Water bath, Autoclave (121oC, 15psi), Vortex mixer, Weighing balance, Colony counter, Compound microscope, and Biosafety cabinet.

Media and chemicals

Muller Hinton agar, Nutrient agar, MacConkey agar, Salmonella Shigella agar, CLED agar, TE buffer, 10% SDS, Proteinase K, Phenol chloroform, 5 molar sodium chloride at pH 5.2, Isopropanol, 70% and 95% ethanol, Agarose, ethidium bromide, distilled water, normal saline, glycerol, and standard antibiotics.

Antibiotic/drugs used

Antibiotics used in the study were purchased from a multiple pharmaceutical company. Eleven antibiotics were selected, which are as follows: Amikacin, Amoxicillin, Ciprofloxacin, Gentamicin, Imipenem, Nitrofurantoin, Oxacillin, Pipemidic acid, Polymixin B, Trimethoprim, and Piperacillin/tazobactam.

Methods

Sampling

This study was done on UTI cases of OPD, Medical, Nephrology, Surgical, Main OT, Urology, and ICU wards of Allied hospital Faisalabad by collecting 600 urine samples from March 2019 to August 2019.

Culture and identification of microbes

After sampling was done, the urine specimens were cultured on basic media and differential media, i.e., Salmonella Shigella agar, McConkey agar and CLED agar. Several biochemical tests were performed to check the incidence of these microorganisms.

Antibiotic sensitivity testing

Eleven types of antibiotics were used to check the resistance of these microorganisms. These are listed below in table 2, along with their dosage.

DNA extraction of bacterial sample

DNA samples of *Escherichia coli*, *Klebsiella pneumonia*, and *Pseudomonas aurignosa* were isolated *via* the chemical method.

Agarose gel electrophoresis

Gel electrophoresis was performed to separate DNA by volume-by-volume instead of purification and representation.

Table 1: Antibiotics and their dosage used in the study (Carlier, Noe *et al.,* 2013).

Antibiotics	Dosage (µg)
Amikacin	30
Amoxicillin + Clavulanic acid	10-20
Ciprofloxacin	5
Gentamicin	30
Imipenem	10
Nitrofurantoin	300
Oxacillin	1
Pipemidic acid	20
Polymixin B	300
Trimethoprim	5
Piperacillin/tazobactam	110

Sequencing

All materials needed for sequencing reaction were supplied to the Macrogen sequencing company (Seoul, South Korea). The Macrogen sequencing company used the ABI 3730X1 DNA sequence (applied biosystem, USA) and nucleotide sequencing was done by the Sanger sequencing technique. Eight reactions were done by this method.

STATISTICAL ANALYSIS

SPSS version 22.0 was used for the calculation of %.

RESULTS

Different media were used to cultivate the microbes and support the development of bacterial organisms as they comprised several nutrients essential for their growth. Table 2 describes the growth of the microbes that appeared in the form of colonies observed regarding the color, size, elevation, margins, texture, shape and consistency and noted as shown in the table.

After culturing microbes on different growth media, several biochemical tests were done to categorize the bacterial colonies. All biochemical testing performed using hospital applied technique. Table 3 presents the results of these tests.

The results of various types of antibiotics used to check the resistance of these microorganisms are presented below in tables 4 and 5. Figs. 1 and 2 depict the pictorial representation of antibiotic sensitivity testing.

Primer information

All materials needed for sequencing reaction were supplied by the Macrogen sequencing company (Seoul, South Korea). The Macrogen sequencing company used ABI 3730X1 DNA sequence (applied biosystem, USA) and the nucleotide sequencing was done by Sanger sequencing technique. Eight reactions were done by this method.

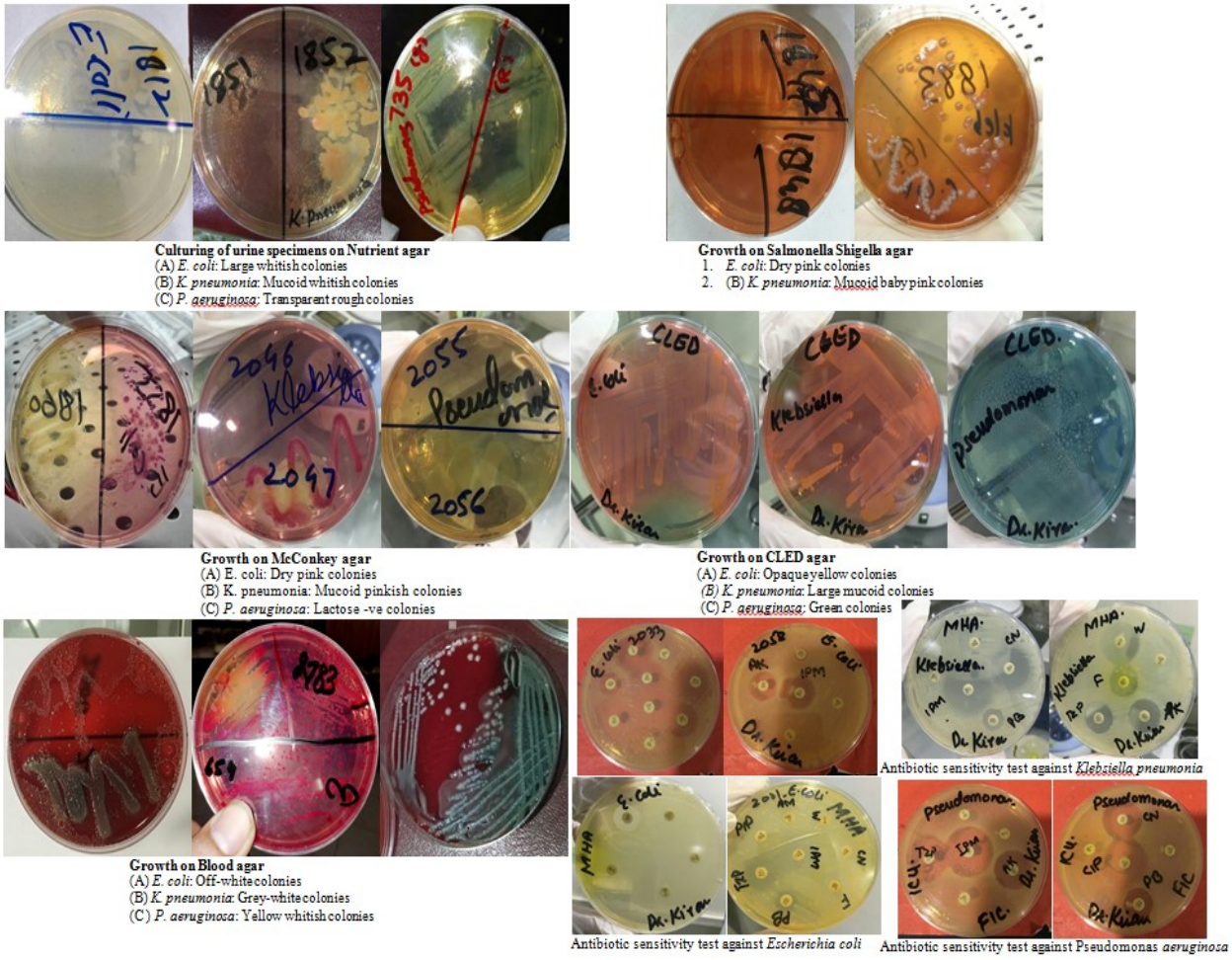

Fig. 1: Sensitivity test on different agar

Table 2: Isolation and identification of different strains of bacteria, isolated from urine samples.

Bacteria	Nutrient agar	MacConkey agar	Salmonella Shigella agar	CLED agar
Escherichia coli	Large whitish colonies	Dry pink colonies	Dry pink colonies	Opaque yellow colonies
Klebsiella pneumonia	Mucoid whitish colonies	Mucoid pinkish colonies	Mucoid baby pink colonies	Large mucoid colonies
Pseudomonas aeruginosa	Transparent colonies rough	Lactose –ve colonies	No growth	Green colonies

Table 3: Result of different biochemical tests of bacterial strains isolated from urine specimens.

Test	*Eschericia coli*	*Klebsiella pneumoniae*	*Pseudomonas aeruginosa*
Catalase test	+	+	+
Coagulase test	-	-	-
Methyl red test	+	-	-
Oxidase test	-	-	+
Simmon citrate test	-	+	+
Urease test	-	+	-
Indole test	+	-	-

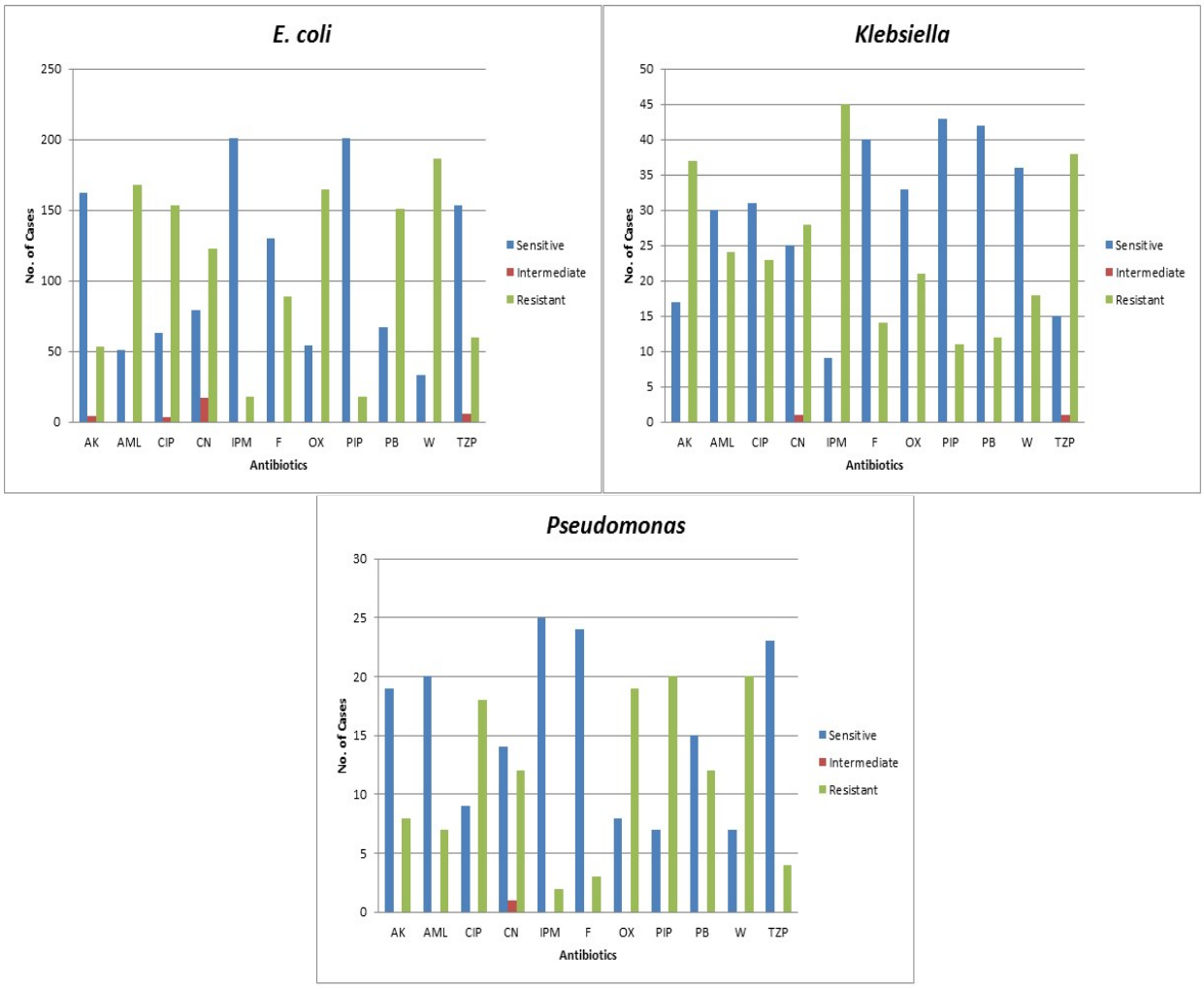

Fig. 2: Sensitivity and resistant data of *E. coli*, *Klebsiella* and *Pseudomonas*

Table 4: Susceptibility pattern of antibiotics regarding the zone of inhibition against isolated uropathogens

Antibiotics			*E. coli* (mm)	*Klebsiella* (mm)	*Pseudomonas* (mm)
Name	Codes	Disks (µg)			
Amikacin	AK	30	12	18	22
Amoxicillin + Clavulanic acid	AML	10-20	-	-	-
Ciprofloxacin	CIP	5	-	-	29
Gentamicin	CN	30	13	22	20
Imipenem	IPM	10	11	28	26
Nitrofurantoin	F	300	20	17	-
Oxacillin	OX	1	-	-	-
Pipemidic acid	PIP	20	-	-	-
Polymixin B	PB	300	11	11	16
Trimethoprim	W	5	-	23	-
Piperacillin/tazobactam	TZP	110	18	18	25

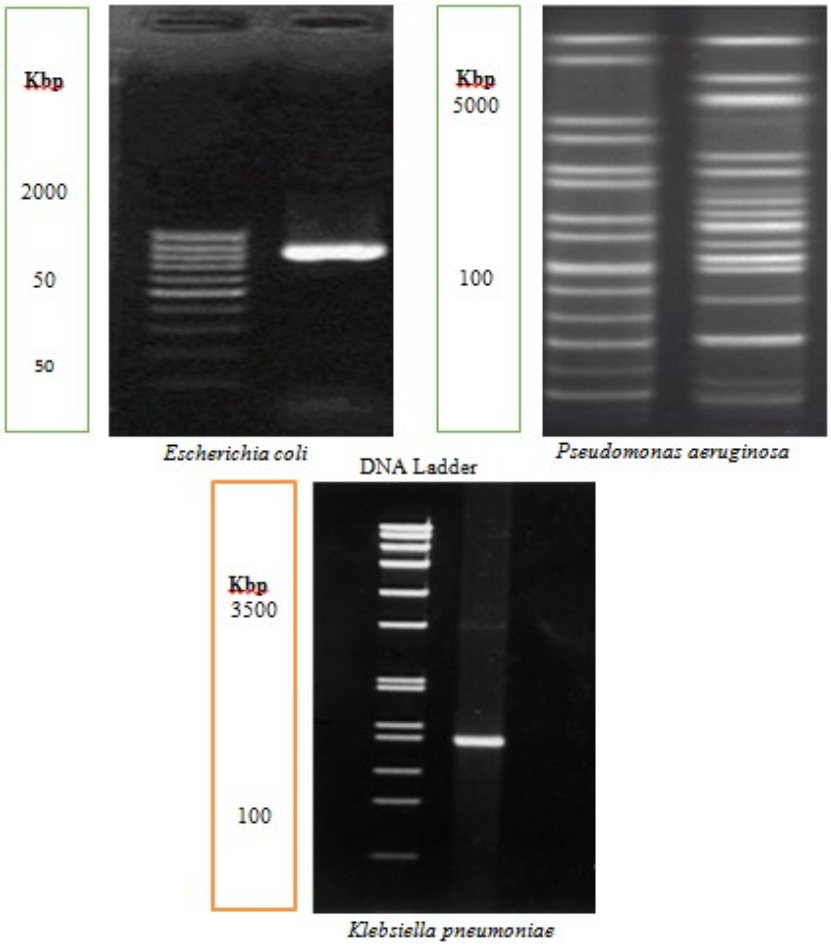

Fig. 3: Confirmation of isolated DNA on Gel electrophoresis (a) *E. coli* (b) *K. pneumoniae* (c) *P. aeruginosa*

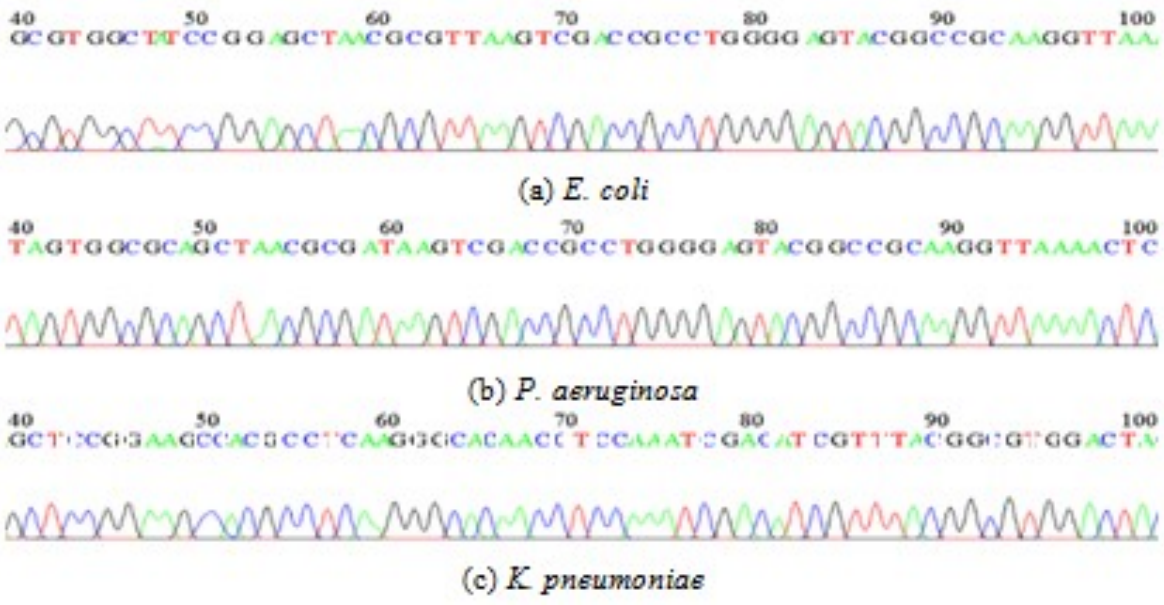

Fig. 4: Trace file analysis.

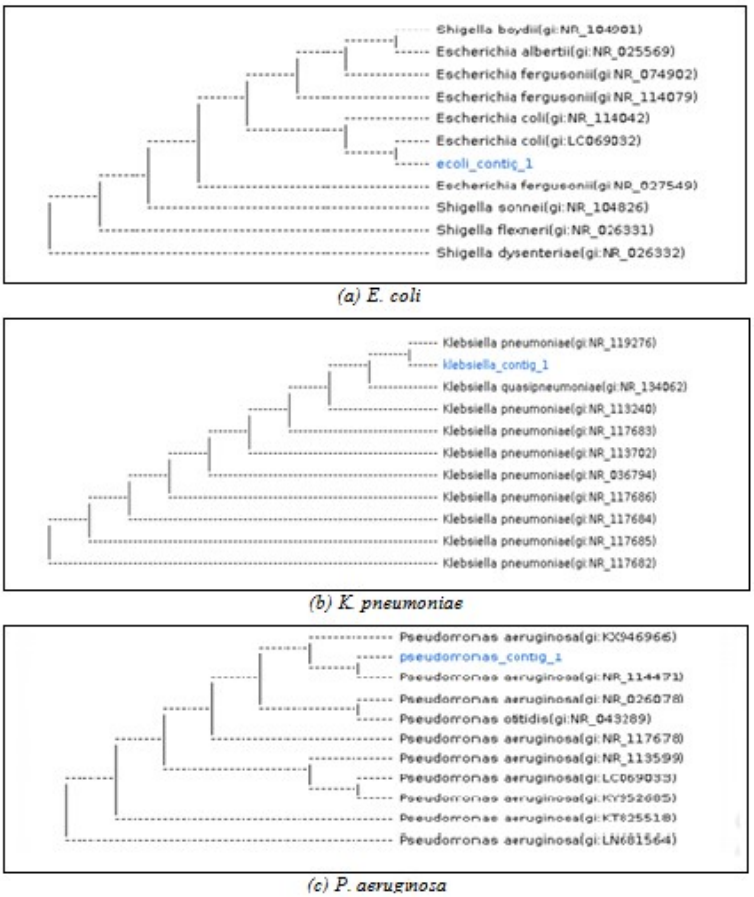

Fig. 5: Phylogenetic tree analysis.

Table 5: Antimicrobial patterns of most frequently isolated gram-negative uropathogens.

No	Antimicrobial Agents	*E. coli* N=219, n%			*Klebsiella* N=54, n%			*Pseudomonas* N=27, n%		
		S	IS	R	S	IS	R	S	IS	R
1	AK	162 74%	4 2%	53 24%	17 31%	-	37 69%	19 70%	-	8 30%
2	AML	51 23%	-	168 70%	30 56%	-	24 44%	20 74%	-	7 26%
3	CIP	63 29%	3 1%	153 70%	31 57%	-	23 43%	9 33%	-	18 67%
4	CN	79 36%	17 8%	123 56%	25 46%	1 2%	28 52%	14 52%	1 4%	12 44%
5	IPM	201 92%	-	18 8%	9 17%	-	45 83%	25 93%	-	2 7%
6	F	130 59%	-	89 41%	40 74%	-	14 26%	24 89%	-	3 11%
7	OX	54 25%	-	165 75%	33 61%	-	21 39%	8 30%	-	19 70%
8	PIP	201 92%	-	18 8%	43 80%	-	11 20%	7 26%	-	20 74%
9	PB	67 31%	-	151 69%	42 78%	-	12 22%	15 56%	-	12 44%
10	W	33 15%	-	186 85%	36 67%	-	18 33%	7 26%	-	20 74%
11	TZP	153 70%	6 3%	60 27%	15 28%	1 2%	38 70%	23 85%	-	4 15%

Primer Information

Sequencing Primer Name Primer Sequences	PCR Primer Name Primer Sequences
785F 5' (GGA TTA GAT ACC CTG GTA) 3'	27F 5' (AGA GTT TGA TCM TGG CTC AG) 3
907R 5' (CCG TCA ATT CMT TTR AGT TT) 3'	1492R 5' (TAC GGY TAC CTT GTT ACG ACT T) 3'

Figure 3.10 Primer sequence information 785F5' to 3' and 907R5' to 3' and PCR primer sequence 27F5' to 3' and 1492R5' to 3'.

Confirmation of isolated DNA on gel

Confirmation of selected DNA bacteria was done through gel electrophoresis. The electrophoresis procedure was carried out in the department of the University of Lahore. To check the result, the gel was presented to ultraviolet light, and the image taken with a gel documentation framework system. These images are shown in fig. 3.

Trace file analysis

Fig. 4 shows a trace file analysis of 40 to 100 nucleotides with peaks of different colors which indicate a different pattern. Black peak describes the value of Guanine; Red describes thiamine, Blue describes cytosine and Green describes adenine.

Phylogenetic tree analysis

Phylogenetic tree analysis shows the developmental connections among the various life forms. In fig. 5, trees were constructed using Mega X software, which deduces the developmental connections between *E. coli*, *K. pneumoniae, P. aeruginosa,* and their related species.

DISCUSSION

UTI incidence is very high among hospitalized, catheterized, and chronically-ill patients (Bouchillon, Badal *et al*., 2013). In Pakistan, people who suffer from UTIs mostly belong to low socio-economic status, poor housing and unhygienic conditions.

The *E. coli* was found as the most isolated uropathogen that is (73%), *Klebsiella pneumonia* (18%) and *Pseudomonas aurignosa* (9%). This study also disclosed that *E. coli* was the most prevalent bacteria among UTI patients.

The isolated strains of uropathogens were processed for testing antibiotic susceptibility against Amikacin, Amoxicillin, Ciprofloxacin, Gentamicin, Imipenem, Nitrofurantoin, Oxacillin, Pipimidic acid, Piperacillin tazobactam, Polymyxin B, Trimethoprim.

In this present study, the most useful antibiotics were Imipenem, Nitrofurantoin, Pipimidic acid, Piperacillin tazobactam, Polymyxin B. These findings correspond to the susceptibility pattern of these drugs. This resistance is due to unnecessary and without prescription use of antibiotics among UTI patients (Petca, Mareș *et al*., 2020).

The *E. coli* isolated from positive urine samples of UTI patients showed susceptibility to various antibiotics. The total of 162 samples were susceptible to Amikacin, 51 to Amoxicillin, 63 to Ciprofloxacin, 79 to Gentamicin, 201 to Imipenem, 130 to Nitrofurantoin, 54 to Oxacillin, 201 to Pipimidic acid, 153 to Piperacillin tazobactam, 67 to Polymyxin B, and 33 to Trimethoprim. In various countries, amikacin is more active against quinolone-resistant *E. coli* than any other aminoglycosides.

The second most prevalent uropathogen was *Klebsiella pneumonia* (Noman, Zahan *et al*., 2020). The strains of *Klebsiella pneumonia* were susceptible as follows: 17 samples to Amikacin, 30 to Amoxicillin, 31 to Ciprofloxacin, 25 to Gentamicin, 8 to Imipenem, 40 to Nitrofurantoin, 33 to Oxacillin, 43 to Pipimidic acid, 15 to Piperacillin tazobactam, 42 to Polymyxin B, and 36 to Trimethoprim.

The *P. aeruginosa* strains isolated from UTI patients were susceptible as: 19 samples to Amikacin, 20 to Amoxicillin, 9 to Ciprofloxacin, 14 to Gentamicin, 25 to Imipenem, 23 to Nitrofurantoin, 8 to Oxacillin, 7 to Pipimidic acid, 23 to Piperacillin tazobactam, 15 to Polymyxin B, and 7 to Trimethoprim.

E. coli has been reported as the most frequent uropathogen with 24% resistance towards Amikacin, 77% to Amoxicillin, 70% to Ciprofloxacin, 56% to Gentamicin, 8% to Imipenem, 41% to Nitrofurantoin, 75% to Oxacillin, 8% to Pipimidic acid, 27% to Piperacillin tazobactam, 69% to Polymyxin B, and 85% to Trimethoprim.

For molecular characterization, the DNA samples of *E. coli*, *K. pneumonia,* and *P. aurignosa* were sequenced with different primers. Analysis and comparison of DNA sequences were performed by trace file analysis and phylogenetic tree analysis, which showed the revolutionary connections of these microorganisms with their related species.

CONCLUSIONS

The most prevalent isolated uropathogens were *E. coli, Klebsiella pneumonia* as well as *Pseudomonas aurignosa* among gram-negative bacteria. Among these mentioned microbes, *E. coli* exhibited the greatest sensitivity to imipenem and pipimidic acid. Whereas *K. pneumonia* displayed the highest sensitivity to pipimidic acid, polymixin B, and nitrofurantoin. *Pseudomonas aurignosa* indicated sensitivity for imipenem, piperacillin-tazobactam, nitrofurantoin. Isolated DNA of these species was confirmed through gel electrophoresis and the results of phylogenetic tree analysis showed a significant connection of these microorganisms with their related species.

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