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MOLECULAR CHARACTERIZATION AND MUTATIONAL ANALYSIS OF AMINOGLYCOSIDES, SULFONAMIDES AND QUINOLONES RESISTANT GENES OF *ESCHERICHIA COLI* ISOLATED FROM UTIS PATIENTS

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Abstract
Antibiotic resistance produced by Gram-negative bacteria causing UTIs is an issue of concern for clinicians which limits the treatment options. The current study is designed to determine the molecular characterization and mutational analysis of antibiotic resistant genes of *E. coli* isolated from UTIs patients. A total of 93 *E. coli* isolates were obtained from UTIs patients at Khyber Teaching Hospital (KTH), Peshawar. The collected samples were identified phenotypically using API 20E strips. The antibiotic resistant genes of *E. coli* were detected by PCR and subsequently sequenced by Next Generation Sequencing (NGS) for mutational analysis. The antibiogram results revealed that most of the *E. coli* isolates were resistant against antibiotics; Ampicillin, cephalosporins, cotrimoxazole, aminoglycosides and ciprofloxacin while showed good results against TGC, MEM, TZP and SCF. Molecular analysis showed that 21 isolates were positive for *Sul1* gene, *Aac1* 10, *Qnrs* 7 and *Sul2* gene 6. The mutational analysis of resistant genes showed that *Aac1* gene have two amino acid substitutions (M120T and R197I) while *Qnrs, Sul1* and *Sul2* have single amino acid substitution (H95R, H292L and E66A), respectively. Different mutations in antibiotic resistant genes; *Aac1*, *Qnrs*, *Sul1* and *Sul2* and *EaeA* of *E. coli* prove that bacteria have a drastic internal machinery modification which can lead to antibiotic resistance.

Keywords: *E. coli*, antibiogram, PCR, resistant genes, NGS, mutational analysis.

INTRODUCTION

Escherichia coli is one of the important Gram-negative member of Enterobacteriaceae, present in human intestine as normal flora. (Röderova *et al.*, 2017)**.** Some species of *E. coli* caused different types of infection while some are non-pathogenic. The pathogenic *E. coli* are categorized into various pathovars and each of them causes different diseases. The pathogenic *E. coli* are responsible to cause disease in intestine and extra intestine infection, e.g., in the intestine causes disorders in the gut

ranging from mild diarrhoea to severe colitis and extra-intestine infections includes urinary tract infections (UTI) and septicaemia (Leimbach *et al.*, 2013).

Antimicrobial resistance is a globally known hazard to health. The impact of primary healthcare is of great consequence as this is where nearly 80% of the entire antibiotics consumed within the health service are advised (Majeed *et al.*, 1999). Bacterial infections resistant to antibiotics can restrict the accessibility of effective treatment alternatives, making some regularly bacterial infections challenging to handle, with urinary tract infections included (Holmberg *et al.*, 1987). In 3rd world countries, the use of latest broad spectrum antibiotics is restricted by affordability of second line drugs as well as reduced access to healthcare, resulting in rising concerns for amplified morbidity and mortality from antibiotic resistant infections in these nations (Planta, 2007).

Almost 150 million people worldwide are infected with UTIs (Harding *et al.*, 1994). Both men and women may become infected, but gender is the predisposing factor for UTIs, so females are more affected as compared to males (Foxman, 2014). Bacteria cystitis lead to UTIs in approximately 25% of women within 6 months (O'Brien *et al.*, 2015). Due to resistance of pathogen to available antibiotics, drug resistance increasing day by day. Similarly, drug resistance among uropathogens were also observed and due to which recurrences of disease occur (Al-Badr *et al.*, 2013; Gupta *et al.*, 2001). Almost 70–90% of all UTIs has been caused by *E. coli* (Gurevich *et al.*, 2016). The cure of patients with antibiotics has become difficult because of the drug resistance among pathogens (Can *et al.*, 2015) The increased resistance against fluoroquinolones and aminoglycosides is due to production of ESBL by *E. coli* (Bonelli *et al.*, 2014). The high prevalence of UTIs has been reported from *E. coli* in the community (Gonçalves *et al.*, 2016).

In addition, by molecular biological techniques 29 virulence factor genes of *E. coli* have been identified (Johnson *et al.*, 2000). It was fond the all the patient of UTI whether they are non-immunocompromised patient, immunocompromised or renal disease patient (Kärkkäinen *et al.*, 2000). It was observed that less virulent *E. coli* can cause UTI in diabetic patient, patients having renal disease and immunocompromised patients (Brauner *et al.*, 1995). The *E. coli* clone are different from each other due to the difference in their serotypes i.e., O and K serotypes, membrane protein and blood degrading enzymes (Tullus *et al.*, 1984). The etiology of UTI caused by *E. coli* (O15:K52:H1) has been studied in Spain recently (Prats *et al.*, 2000). There was only 1.4% of UTIs and pyelonephritis caused by *E. coli*.

It is therefore necessary to determine the antibiogram and molecular characteristics of resistant bacteria in the hospital settings to take infection control measures and empirical treatment for infections. The current study determined the Evaluation of aminoglycosides, sulfonamides and quinolones resistance genes in *E. coli* and their antibiogram isolated from UTIs patients in tertiary care hospital Peshawar. This will be helpful for clinicians to overcome the resistance mechanisms.

MATERIALS AND METHODS

Study design

The current research work was conducted at the research laboratory (COBAM), University of Peshawar and Department of Pathology, Khyber Teaching Hospital (KTH), Peshawar. The study was approved by the Institution Research and Ethical Review Board (IREB) of Khyber Medical College, Peshawar (Document No. 122/ADR/KMC). The study was approved by the Institution Research and Ethical Review Board (IREB) of Khyber Medical College, Peshawar (Document No. 122/ADR/KMC).

Sample collection and identification

A total of 93 resistant clinical isolates were obtained from urine in sterile bottles from both outdoor and admitted patients having Urinary Tract Infections (UTIs) visiting to KTH, Peshawar. The collected samples were inoculated on CLED media and incubated overnight at 37°C for bacterial growth. These bacterial colonies were then subjected to Gram staining technique to differentiate Gram positive and Gram-negative isolates. The urine isolates were identified as *E. coli* using API (Analytical Profile Index) 20E strip. The antibiogram of identified isolates was performed by Baur and Kirby method (disc diffusion) using Muller Hinton Media (MHA). Then these isolates were preserved in Tryptone soya broth (TSB) media with 15% glycerol and subsequently stored at -80°C (Kurazono *et al.*, 2003) for further molecular analysis.

DNA Extraction

DNA extraction was performed by using old broth cultures (24- 48 hours) through Thermoscientific Gene JET Genomic DNA Kit. After extraction, the DNA was checked through 1% Gel by electrophoresis. The DNA was preserved at -20°C.

Molecular characterization of antibiotic resistant genes

The selected antibiotic resistant genes were amplified by using conventional Polymerase Chain Reaction (PCR) machine (Labnet, Labnet International, Inc. USA). The specific primers were used for the amplifications of *Sul1*, *Sul2*, *Aac1* and *QnrS* genes shown in Table 5**.** The condition for each sample in PCR was optimized. The reaction was processed by mixing 0.5uL of each reverse and forward primers (oligonucleotides, Macrogen Korea), 11.5uL of Nuclease-free water, 12.5uL Taq Master Mix (Bioron, life sciences), and 2uL of sample DNA. The known sample and PCR mixture with no template was used as positive control and Negative control respectively. Different PCR conditions were applied for different genes shown in **Table 1.**

Gel electrophoresis

The amplicon was run on 2% agarose gel electrophoresis (Bioron, Life sciences) and bands were visualized through gel documentation system (BIO-RAD Gel Doc™ XR+). The amplicon sizes were determined by matching with DNA ladder (100bp). These PCR products were further subjected to sequencing for mutational analysis after purification.

Mutational analysis of antibiotic resistant genes

The PCR products of resistant genes were randomly selected and purified for sequencing process with a PCR Purification Kit (Thermo Scientific, USA). The products were sequenced directly by Next Generation Sequencing (NGS) at Genomic Sciences, Rehman Medical Institute (RMI) Peshawar. The FASTA sequences of PCR products were matched with the original gene sequences of GenBank (NCBI database). The amino acid and nucleotide sequences were analyzed by searching the database with BLAST and BioEdit Software. The IBM SPSS Statistics (version 23.0.0) software was used for the calculation of frequencies and percentages in the current study. The Origin (version 2018) was used for plotting different graphs of frequencies and percentages in different isolates of the present research work.

RESULTS

A total to 93 clinical urine isolates yielded the growth of *E. coli* and were selected for the current study on basis of resistance profile using phenotypic methods. Out of 93 urine isolates, 44 (47.31%) were collected from male patients while 49 (52.68%) isolates were obtained from females. Among 93 clinical isolates of *E. coli*, the highest prevalence was recorded in the age group of 41-60 (45.1 %), followed by the age groups 21-40 years (33.3%), 11-20 years (8.6%) and 0-10 (4.3 %). Among all these age groups, the lowest prevalence was observed in the age group of 0-10 which is (4.3 %) as shown in **Table 2**.

All the *E. coli* isolates were tested against 18 selected antibiotics by disc diffusion method using CLSI-2021 guidelines. The antibiogram results revealed that most of the *E. coli* isolates were resistant against antibiotics; Ampicillin, cephalosporins, cotrimoxazole, aminoglycosides and ciprofloxacin while showed good results against TGC, MEM, TZP and SCF. as shown in the **Table 3**.
The current study reported the molecular characterization of different targeted antibiotics resistant genes in clinical isolates of *E. coli* obtained from UTIs infected patients. The results of study revealed that 21 (22.58%) isolates contained *Sul1* gene, *Sul2* gene in 6 (6.25%), *Aac1* in 10 (10.75%) and 7 (7.52%) isolates contained *QnrS* gene as shown in **Figures 1-2**. The current study reported different antibiotic resistance genes combination among clinical isolates of *E. coli.* The results revealed that 7 isolates had two antibiotic resistance gene combination (*Sul1 + Aac1, Sul1 + QnrS and Sul2 + QnrS*) and 46 isolates have one antibiotic resistance gene (*Sul1, Sul2, QnrS, Aac1*). While 40 isolates have no antibiotic resistance gene as shown in **Table 4.**

The current experimental work was further subjected to NGS sequencing for mutational study. Among the sulphonamides, aminoglycosides and quinolones resistant *E. coli* isolates some isolates for each gene were randomly selected for genetic characterization of the *Sul 1, Sul 2, Aac 1* and *Qnrs* gene by sequencing process. Finally, these sequences were compared with the published *sul 1, sul 2, Aac 1* and *qnrs* sequences of *E. coli* strains R 46, EC332, C1984 and MN067 in the gene bank with the accession numbers: KU603647.1, NG_048081.1, NG_052506.1 and NG_059276.1 respectively. These data were analyzed by using Bio Edit software.

Out of 93 clinical isolates only 10 isolates carried out resistance gene (table of genotypic conformation). From these 10 isolates *Aac 1* positive isolates were randomly selected for mutational study. The result show that both isolates have an amino acid substitution at position M120T and R197I as shown in the following **Table 5**. In synonymous mutation the nucleotide change was observed at position 402 and 405. This alteration has no effect on amino acid substitution as shown in **Table 5**.
The result of the current study showed that 7 isolates out of 93 only (7.52%) were positive for *qnrs* gene. The result of mutational analysis showed that both isolates had an amino acid substitution at only position number H95R as shown in the following **Table 5**. No synonymous mutation was found in this gene. The result of the current study showed that out of 93 isolates only 21 isolates carried out resistance gene. For mutational study we select these 21 samples randomly. The result showed that both isolates have an amino acid substitution at position number H292L (**Table 5**). No synonymous mutation was found in this gene.
Out of 93 isolates 6 isolates carried out *sul 2* resistant gene. From these two isolates were randomly selected for mutational study. The result of mutational study showed that both isolates have an amino acid substitution at position E66A as shown in the following table (**Table 5**). There is no synonymous mutation was found in this gene.

DISCUSSION

E. coli is found universally found in the gastrointestinal tract of human as normal flora but sometimes occasionally, it becomes pathogenic and can cause diseases like urinary tract infection (UTIs), bacteraemia and wound infections (Karlowsky *et al.,* 2002). UTIs are frequently caused infections in children and adults and can produce adverse effects (Asokan *et al.*, 2017). Previously conducted studies have reported that *E. coli* is the main pathogen causing UTIs (Karlowsky *et al.*, 2002). The current study was conducted to determine the antibiotics resistant *E. coli* among patients visiting KTH hospital, Peshawar. A total of 93 antibiotics resistant isolates were collected among which 49 (52.68%) were obtained from females while 44 (47.31%) were recovered from male patients. The incidence rate of *E. coli* in this study was higher in females (52.68%) as compared to male patients. Our results are in line with the study conducted by (Odoki *et al.*, 2019) where (40.9%) female were effected with UTIs. Possible reason for increased prevalence of *E. coli* among females is due to the contiguity of anus to vagina.

The current study reported the frequency distribution of resistant *E. coli* isolates causing UTI infection among different age groups. In our study, highest prevalence of *E. coli* was observed in age group of 41-60 years (45.1%). Our results are on same line with a study conducted by (Karishetti *et al.*, 2019) where increased prevalence was reported in age group 41-60 (25.4%).

Antibiotics play an important role in the treatment of different life-threatening infections but emergence of antibiotics resistance genes by the bacteria has altered the scenario. Antimicrobial resistance is frequently reported by scientists all over the world (Reller *et al.*, 2009).

Routinely, antibiotics are considered powerful tools for the treatment of UTIs. But consistent and misuse of antibiotics are the main cause of bacterial resistant towards drugs. This study reported a total of 18 routinely used antibiotics against *E. coli* causing UTIs. Against TZP and SXT; 85(91%) and 78 (84%) resistance was observed in resistant *E. coli* isolates respectively. The clinical isolates also showed high resistance against quinolones antibiotics like CIP 69(74%) and LEV 66(71%).

High level of sensitivity of cephalosporins and carbapenems have also been reported by (Adib *et al.*, 2014). The result of the antibiogram revealed that the antibiotics like tygacil and colistin showed good results i.e. (100%) sensitive against all the resistant isolates of *E. coli*.

In our study, (22.58%) isolates showed resistance against *sul*1 gene, while 10.75% isolates were found resistant to *Aac* 1 gene. However, only 7% and 6% isolates showed resistance towards *Qnrs* and *sul*2 respectively. The study conducted by (Hammerum *et al.*, 2006) showed increased prevalence of *sul*1 (55%) gene in Denmark which is higher as compared to our findings. Another study also reported the increased prevalence of sul2 which are increased than our results. Possible reason for this inconsistency might be due to the dissemination of multiple resistance genes along with extended spectrum β-lactamases in *E. coli.*

In our study resistant isolates of *E. coli* were further subjected to NGS for mutational analysis. Previously conducted studies have shown that amino acid substitutions in case of *Qnr*S can reduce quinolone activity. The same findings were also reported in our study that amino acid substitution at position 95 *Qnr*S gene expressed resistance to quinolone (Cattoir *et al.*, 2007).

Similarly, amino acid substitutions have also been observed in *sul*1/*sul*2 genes in *E. coli* at position number H292L and E66A respectively. These substitutions have resulted in the emergence of sulphonamides resistance.

Documented evidence has shown increased prevalence of antibiotics resistance in the infections caused by *E. coli*. Therefore, it is utmost important to raise awareness among local community about the rationale use of antibiotics to combat rising resistance. It is also necessary to study the genetic background of pathogen to gather sufficient information about mutations and resistance.

CONCLUSIONS

The high spread of resistance mechanism among the bacteria is an issue of concern for clinicians during treatment management. The multidrug resistant *E. coli* is highly spreading among the hospitalized and non-hospitalized patients resulting life threatening infections. The current study documented that the *E. coli* isolates were resistant against antibiotics; Ampicillin, cephalosporins, cotrimoxazole, aminoglycosides and ciprofloxacin. Molecular analysis showed that 21 isolates were positive for *Sul1* gene, *Aac1* 10, *Qnrs* 7 and *Sul2* gene 6. The mutations (M120T and R197I) were observed in *aac1* gene while *qnrS, sul1* and *sul2* have single amino acid substitution (H95R, H292L and E66A), respectively. Different mutations in antibiotic resistant genes; *Aac1*, *Qnrs, Sul1* and *Sul2* and *EaeA* of *E. coli* prove that bacteria have a drastic internal machinery modification which can lead to antibiotic resistance.

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Table 1: Oligonucleotide primer sequences used for the molecular detection of antibiotic resistance genes of *E. coli*.

Targeted Genes	Specific Primers	Product Size (bp)	Annealing Temperature	Cycles
Aac 1	**F**' TTGCGATGCTCTATGAGTGGCTA **R**' CTCGAATGCCTGGCGTGTTT	482	57^{O}C for 30 sec	30
Sul1	**F**' TTCGGCATTCTGAATCTCAC **R**' ATGATCTAACCCTCGGTCTC	822	58^{O}C for 30 sec	30
Sul2	**F**' CCTGTTTCGTCCGACACAGA **R**' GAAGCGCAGCCGCAATTCAT	435	53^{O}C for 30 sec	30
QnrS	**F**' TCATACATATCGGCACCGCA **R**' ACAATACCCAGTGCTTCGAGA	627	52 ^{O}C for 30 sec	30

Table 2: Frequency of different age groups and gender wise distribution of *E. coli* isolates

Gender	Frequency	Percent (%)
Male	44	47.31
Female	49	52.68
Different age groups		
0-10	4	4.3
11-20	8	8.6

21-40	31	33.3
41-60	42	45.1
>60	8	8.6
Total	93	100

Table 3: Antibiogram of *E. coli* isolated from UTI infected patients.

	Symbols	Sensitive (n)	Percentage (%)	Resistant (n)	Percentage (%)
Amoxicillin – Clavulanate	AMC	61	65.6	32	34.40
Ampicillin	AMP	14	15.0	79	84.9
Cefoperazone-sulbactam	SCF	74	79.5	19	20.4
Piperacillin-Tazobactam	TZP	67	72.0	26	28.0
Cefepime	FEP	69	74.1	24	25.9
Cefotaxime	CTX	51	54.8	42	45.1
Ceftazidime	CAZ	53	57	40	43
Aztreonem	ATM	55	59.1	38	40.8
Meropenem	MEM	87	93.5	6	6.45
Amikacin	AK	43	46.2	50	53.7
Gentamycin	CN	30	32.2	63	67.7
Doxycycline	DO	40	43	53	57
Ciprofloxacin	CIP	28	30.1	65	69.9
Levofloxacin	LEV	30	32.2	63	67.7
Cotrimoxazole	SXT	18	19.3	75	80.6
Chloramphenicol	C	64	68.8	29	31.1
Fosfomycin	FOS	80	86.0	13	14.0
Tygacil	TGC	93	100	00	00

Table 4: Different antibiotic resistance genes combination among clinical isolates

Antibiotic resistance genes	**Frequency**	**Percentage (%)**
Sul 1	22	23.65
Sul 2	06	6.45
Aac 1	10	10.75
Qnrs	08	8.60
Sul 1, Aac 1	02	2.15
Sul 1, Qnrs	03	3.22
Sul 2, Qnrs	02	2.15
No Genes	40	43.01

Table 5: Synonymous and non-synonymous mutations of aminoglycosides, sulfonamides and quinolones resistance genes of *E. coli* isolates

Nucleotide position	**Reference amino acid**	**Altered amino acid**	**Amino acid position**
Non synonymous mutation of gene Aac 1			
359	M (ATG)	T (ACG)	120
590	R (AGA)	I (ATA)	197
Synonymous mutation of gene Aac 1			
402	T → A	P	134
405	C → T	V	135
Non synonymous mutation of gene Qnrs			
284	H (CAC)	R (CGC)	95
Non synonymous mutation of gene Sul1			
875	H (CAT)	L (CTT)	292
Non synonymous mutation of gene Sul2			
197	E (GAC)	A (GCG)	66

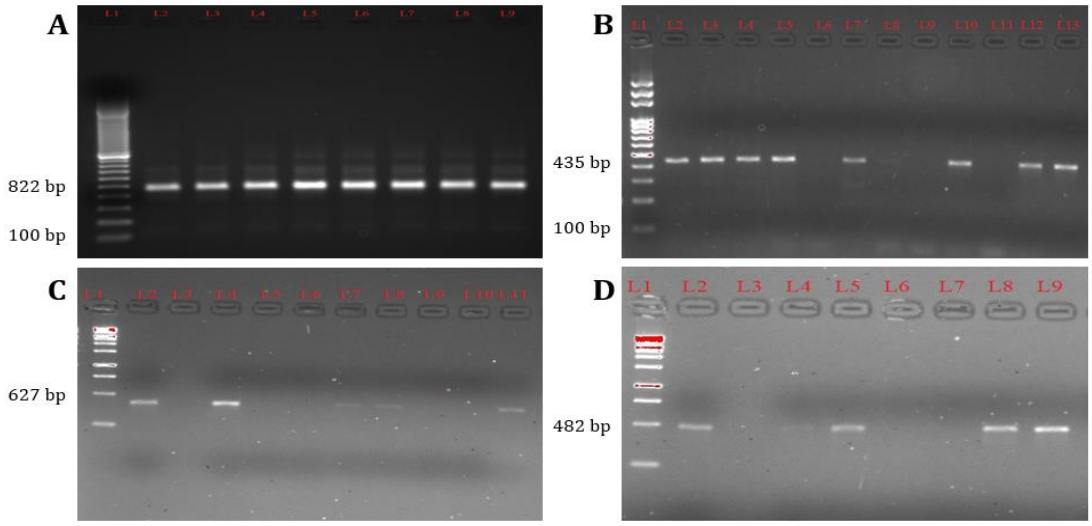

Figure 1: Gel electrophoresis of antibiotic resistant genes of *E. coli*; A) Sul1(822bp), B) Sul2 (435bp), C) QnrS (627bp) and D) Aac1 (482bp).

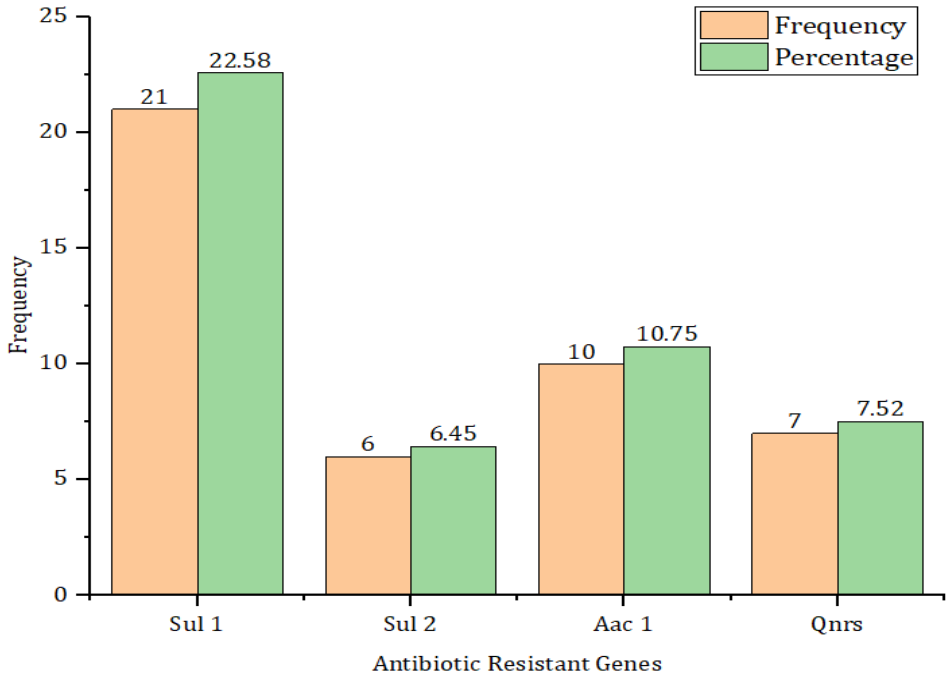

Figure 2: Distribution of Antibiotic resistance genes of *E. coli* obtained from UTIs patients