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Research Article

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Prevalence of Quinolone Resistance Genes gyrA and gyrB in Bacteria Isolated from Urinary Tract Infections

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ABSTRACT

Introduction: Antibioticshave significant importance in medicinal drugs but unfortunately bacteria can adapt various resistance mechanisms against those due to their abuse. A frequent way through which nitrate reducing uropathogens acquire resistance mechanism against fluoroquinolones group of antibiotics is by continuously occurring mutation either in gyrA or gyrB genes through altering enzyme DNA gyrase that help in DNA replication. Microorganisms causing Urinary Tract Infection (UTI) vary in their susceptibility to antimicrobials from place to place and from time to time.

Aim: The current study was performed to find the prevalence of nitrate reducing bacteria in district Kohat and Hangu, their antimicrobial sensitivity pattern to various fluoroquinolones group of antibiotics and detection of mutation in gyrA and gyrB genes in the resistant bacterial isolates. Nitrate reducing bacteria i.e *E.coli, Klebsiella* and *Proteus* areconsidered to be the frequent cause of UTI thatreduce nitrate to nitrite by releasing enzyme nitrate reductase.

Methodology: A Total of 252 urine specimens of suspected UTI patients were collected from Districts Head Quarter Hospitals (DHQ) and Combined Military Hospital (CMH) hospitals of districts Kohat and Hangu, Pakistan. Specimens reactive to nitrate strip reduction test were inoculated on (Cysteine Lactose Electrolyte Deficient) CLED media, incubated at 37°C for 24 hours. Ciprofloxacin, levofloxacin, norfloxacin, ofloxacin and moxifloxacin fluoroquinolones group of antibiotics were used for to check their sensitivity patterns.

Result: One hundred and seventy-five (69.44%) urine specimens were positive for nitrate reduction test, including 135 (77.14%) *E. coli*, 33 (18.85%) *Klebsiella* and 7 (4.0%) *Proteus,* verified through biochemical tests.

Conclusion: Amonge the nitrate reducing bacteria, fifty-one (37.22%) out of 135 *E. coli*, 9 (27.27 %) out of 33 *Klebsiella* and 2 (28.57%) out of 7 *Proteus* were resistant to most of fluoroquninolones group of antibiotics with moxifloxacin being the least resistant while ofloxacin being the most resistant antibiotic Malformed DNA gyrase producing mutated gyrA and gyrB genes were further detected in nitrate reducing bacteria showing resistant to fluoroquninolones.

Key Words: Prevalence, Quinolone resistance, Nitrate reducing bacteria, Proteus, GyrA and gyrB, Urinary tract Infections

INTRODUCTION

Urinary Tract infection (UTI) is the most serious complication which occurs when bacteria accumulate into any passage of the urinary tract, including kidneys, ureters, bladder and urethra. It is one of the most frequent bacterial infections, accounting for about 0.9% of all ambulatory visits in United States.[1] UTI exists both in community and hospital settings. If it is not cured, it may lead to recurrent infection that harm the urinary tract.[2] UTI is one of the most prevalent infectious diseases utterly infecting 150 million people annually world-wide which costs more than 6 billion US dollars.[3] Approximately 3-5% female and 1-2% male develop UTI across the world.[4] Although, UTI affects people of all ages but women are more susceptible than men due to short urethra, pregnancy, absence of

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prostatic secretion and easy contamination of the urinary tract with faecal flora.[5]

The risk factors accompanying UTI include gender, frequency of sexual activity, urethral length, genetic predisposition like blood groups, spermicidal agents usage, rate of urination, personal hygiene and practice of birth control pills, immune suppression, pregnancy, urologic structural abnormalities, diabetes, hypertension, nosocomial infections, and instrumentation like catheterization.[6]

UTI is caused by various bacteria like *Staphylococcus saprophyticus,* Haemolytic *streptococci, Enterococcus faecalis, Neisseria gonorrhoeae, Pseudomonas aeruginosa,* andfungus *Candida* organisms that do not reduce nitrate to nitrite and are non-reactive to nitrate strip reduction test. Among all urinary pathogens, frequently occurring nitrate reducing bacteria (*Escherichia coli*, *Proteus* and *Klebsiella*) release the enzyme nitrate reductase that have the ability to reduce nitrate to nitrite and can be detected simply by dipping a nitrite reagent strip in urine which turns white colour into pink and is a sure indicator of UTI particularly when culture facilities are not available.[7] Amongst nitrate reducing bacteria, *E. coli* is the most common cause of UTI as urine culture and sensitivity report declared them in 85%-95% as causative agent.[8] Signs and symptoms of UTI are dysuria, hematuria, urgency, and bacteriuria. In pyelonephritis, patient grants with flank pain and fever.[9]

The current study explores the prevalence of nitrate reducing uropathogens exhibiting gyrA and gyrB genes in districts Kohat and Hangu, Khyber Pukhtunkhwa, Pakistan.

MATERIALS AND METHODS

Study Area

Patients having UTI were dealt with in the current study for more than eight months. Urine specimens were collected from Districts Head Quarter Hospitals (DHQ) of Kohat, Hangu and from Combined Military Hospital (CMH) of both districts, Pakistan. Sample were then transferred to Department of Microbiology of the university by providing suitable conditions.

Samples Selection Criteria

Patients were advised to pass the midstream urine and to stop antibiotics prior 24 hours of specimen delivery in order to avoid chance of false positive and false negative result respectively.

Ethical Approval

The proposal was approved from the university (KUST) Research Ethics Committee (REC).

Sampling

A total 252 urine specimens were collected. One hundred and twenty-six urine specimens were collected from District Head Quarter Hospital (DHQ) Kohat and CMH Kohat while 126 urine specimens were collected from District Head Quarter Hospital (DHQ) Hangu and CMH Hangu (Thall), which were carried by providing specific conditions to the laboratory of Microbiology Department of the university (KUST).

Sample Culturing

Urine specimens were inoculated on Cysteine Lactose Electrolyte Deficient (CLED) media plate, and then incubated for 24 hours at 37°C under aerobic condition. At last bacterial growth were observed. For preservation, the isolated strains of bacteria were preserved in 30% glycerol at 80 ° C.[10]

Identification of Nitrate Reducing Bacteria

The obtained growths on CLED were identified by visual appearance of colony, string formation, microscopic examination, motility, Gram staining and biochemical diagnosis [23].

Colony Morphology

The bacterial growth obtained on CLED media plates after specific incubation were seen thoroughly for its visual appearance and pointed out its colony color, size, shape, margins, elevation and consistency.

Gram's staining

Gram stain is used to differentiate Gram positive bacteria from Gram negative bacteria. The growth obtained on media were taken with a wire loop or matchstick or sterile ear swab and streaked on slide. The slides were air dried and then were stained with four stains. Firstly, primary stain (crystal violet) was used and washed with tap water after 1 minute. Secondly, mordent (Gram Iodine) for 1 minute and washed again with tap water. Thirdly, decolourizer (ethanol) was poured and slide was then washed after 30 seconds. Lastly, counter stain (safranin) was poured on slide and washed after 45 seconds. The slides were air dried and examined under 100X lens by applying oil on it.

Biochemical and Other Identification Tests

All the nitrate reducing uropathogens were identified through nitrate reduction test, indole production and string formation test.

Nitrate Reduction Strip Test

Nitrate reduction strip test is used for the fast detection of nitrate reducing bacteria. These bacteria release enzyme reductase that reduce nitrate (NO_3^-) in urine to nitrite (NO_2^-). Nitrite further converted to nitrous acid which then reacts with sulfanilic acid reagent (coated on strip) and converts them in diazodide sulfanamide. Further, α- naphthylamine is

an indicator which changes white colour of the strip to pink colour (Figure:1). Test is positive for *E.coli, Klebsiella* and *Proteus*.[11]

Indole Test

This test was specifically performed for the detection of *E. coli* that can release enzyme trytophanase. Little colonies were taken from CLED media and poured into the tubes containing tryptophan and peptone water then allow the tubes for a night incubation at 37°C. After proper incubation, 3-4 drops of Kovac's reagent (4-dimethyl amine benzaldehyde, hydrochloric acid, isoamylalcohol) were added in tubes. The formation of red colour ring on the upper surface of tube within 10 minutes indicates indole production and test is then positive [25].

String Formation Test

Bacteria colonies were taken with help of wire loop and observed string formation which is the specific characteristic of *Klebsiella.*

Glycerol Storage of Bacterial Isolates

30% glycerol was prepared by adding 70ml of distilled water in 30ml glycerol and then was autoclaved. Thirty µl from this solution was mixed with 70µl of bacterial growth suspension in eppendorf tube and was labelled. The tubes were stored at - 80°C for long time preservation and further research.

Analysis of Antibiotic Resistance Pattern

Preparation of Bacterial Lawn

Bacterial colonies were taken from CLED media and made suspension of it in distilled water ampule. A sterile swab was taken and introduced to the bacterial suspension. The swab was then streaked on Muller Hinton (MH) media and soaked. Different fluoroquinolones antibiotics discs were placed on media and given incubation of more than 24 hours. After that Minimum Inhibitory Concentrations (MICs) were read.

Antimicrobial Sensitivity for Nitrate Reducing Uropathogens

Muller Hinton Agar was inoculated with standardized inoculants (0.5 McFarland) using sterilized cotton swab. Fluoroquinolones antimicrobial discs such as ciprofloxacin, norfloxacin, ofloxacin, levofloxacin and moxifloxacin were placed. Subsequently, plates were incubated overnight at 37 °C for 24 hour and then MIC of all fluoroquninolone antibiotics were measured in order to find out their sensitivities.[7]

DNA Extraction

Bacteria were inoculated in nutrient broth and incubated overnight. Then, the collected bacterial cells obtained through centrifugation were washed with 1 ml lysis buffer (Triton X-100). Pellet was obtained by centrifugation at 13,000 x g for 5 minutes. Pellets were suspended in 100 ml of lysis buffer and boiled for 30 minutes. Cell debris were removed using centrifugation at 13,000 x g for 5 minutes. Supernatant was transferred to a new tube. 50 ng of supernatant was used for Polymerase Chain Reaction (PCR).[12]

Molecular Detection of gyrA and gyrB Genes

The gyrA as well as gyrB genes were detected and amplified using PCR, in which the following steps were involved.[13, 14]

Amplification of gyrA and gyrBGenes

The primers was designed by primer3 online software. The gyrA gene was amplified with forward primer 5′(TGTCCGAGATGGCCTGAAGC)3′ and reverse primer 5′ (TACCGTCATAGTTATCCACG) 3′ while the gyrB gene was amplified with forward primer 5′ (CAAACTGGCGGACTGTCAGG) 3′ and reverse primer 5′ (TTCCGGCATCTGACGATAGA) 3′ by PCR.[15]

Polymerase Chain Reaction

Three basic steps were followed for the amplification of the gyrA and gyrB genes.

Initial denaturation in which the template strand was heated to 94°C for two and one minutes respectively resulting in separation of both the strands from one another followed by annealing at 60°C. Extension performed at temperature of 72°C in which new strands were synthesized by taq DNA polymerase using dNTPs to the 3′ end of the primers.

For PCR analysis 25ul volume of reaction mixture was used from each specimen that contained 4ul of 10X PCR buffer, 2.5ul of $MgCl_2$, 2.5ul dNTPs, 0.5ul of ampliTaq DNA polymerase, 1ul of forward primer, 1ul of reverse primer, 3ul of DNA sample and 10.5ul of distilled water.

Polymerase Chain Reaction conditions

The tubes containing master mix were placed in thermal cyclers (ProFlex PCR system). The amplification process was performed accordingly to the given thermo cycling conditions (Table:1).

Gel electrophoresis and visualization of amplified products

The gyrA and gyrB genes amplified products were detected on gel electrophoresis in 1.5% agarose gel containing 0.75 grams of agarose in 50ml of TBE buffer and 5µl ethidium bromide. A molecular weight marker of 100bp was loaded into a well to detect the size of the amplified PCR products. Gel Electrophoresis was performed for 30 minutes at 100 volts. The bands were visualised by the Gel Doc System (Biometra).

RESULTS

Isolation of Nitrate Reducing Bacteria

A total of 252 urine specimens (126 from district Hangu and 126 from district Kohat) were collected from suspected UTI patients, out of which 175 were positive for nitrate reduction strip test (95 from district Hangu and 80 from district Kohat) (Table 2), (Table 3, 4).

Visual Appearance of Urine Specimens Suspected UTI

Centrifugation is performed, sediments were deposited on slide and examine under microscope. Patients with UTI mostly bear cloudy, turbid, translucent and foul smell urine due to bacteriuria, pyuria, proteinuria, may be haematuria and other agents (Figure:2).

Cultural Characteristics and Gram Stain

In order to isolate various nitrate reducing uropathogens from collected urine specimens, CLED agar was used. Specimens were inoculated on CLED media and incubated at 37°C for 24 hours. *Escherichia coli*, *Klebsiella* and *Proteus* growth were obtained. *E. coli* and *Klebsiella* were identified as they ferment lactose on CLED media. *E. coli* produce yellow (lactose fermenting) opaque colonies usually with slightly deeper colour in centre (Figure:3), whereas *Klebsiella* formed large mucoid yellow or yellow white colonies with string formation (Figure:4). *Proteus* gave translucent blue grey colonies (Figure:5). Gram stain were then perfomed from each colony. All Gram-negative bacterial isolates were of pink color (Figure:6).

Motility, Indole and String Formation Test

Motility test for nitrate reducing uropathogens were performed by taking colony from CLED media plates and prepared wet mount slide from it. *E. coli* and *Proteus* were motile while *Klebsiella* were non motile.

Indole and string formation test were also performed for nitrate reducing bacterial isolates (Figure:7).

Antibiotic Susceptibility Patterns

Antibiotic susceptibility patterns for nitrate reducing bacteria such as *E. coli, Klebsiella* and *Proteus* were carried out. Fluoroquinolones group of antibiotics were used. Among them, ciprofloxacin, norfloxacin, ofloxacin, levofloxacin, and moxifloxacin were used. As there were 252 urine specimens from suspected UTI patients in which 175 (69.44%) were positive for nitrate reduction test included 135 *E. coli* (77.14%), 33 *Klebsiella* (18.85%) and 7 *Proteus* (4.0%). Among 135 *E. coli* isolates, 51(37.22%) were resistant to most of fluoroquinolone antibiotics while 09 out of 33 (27.27 %) were resistant to *Klebsiella* isolates and 2 out of 7 (28.57%) *Proteus* isolates were resistant to most of fluoroquinolones group of antibiotics. Among resistant fluoroquinolones antibiotics, levofloxacin was resistant to 49 *E. coli* out of 51 (96.07%), 9 *Klebsiella* out of 9 (100 %) and 2 *Proteus* out of 2 (100%). Ciprofloxacin was resistant to 48 *E. coli* out of 51 (94.11%), 9 to *Klebsiella* out of 9 (100 %) and 2 to *Proteus* out of 2 (100 %). Ofloxacin resitant *E. coli* were 51 out of 51 (100%), 9 *Klebsiella* out of 9 (100%) and *Proteus* were 2 out of 2 (100 %). Norfloxacin resitant *E. coli* were 48 out of 51 (94.11 %), 8 *Klebsiella* out of 9 (88.88 %) and *Proteus* were 2 out of 2 (100 %). Moxifloxacin resitant *E. coli* were 42 out of 51 (82.35 %), 6 *Klebsiella* out of 9 (66.66%) and *Proteus* were 2 out of 2 (100 %). Moxifloxacin was the least while ofloxacin found to be the most resistant antibiotic in fluoroquninolones group (Figure:8, 9, 10, 11), (Table: 5, 6, 7).

Molecular detection of gyrA gene

The bacteria resistant to fluoroquinolones were proceeded further for DNA extraction and molecular detection. The gyrA gene was amplified through PCR machine by designing both forward and reverse primer for it. After amplification, the PCR products were run on gel electrophoresis as shown in figure 12.

Molecular Detection of gyrB gene

The bacteria resistant to fluoroquinolones were proceeded further for DNA extraction and molecular detection. The gyrB gene were was amplified through thermal cycler by designing both forward and reverse primer for it. After amplification, the PCR product were run on gel electrophoresis. The result is shown in figure 13.

DISCUSSION

Urinary Tract infection (UTI) is an infection which occurs when bacteria accumulate into any part of the urinary tract, including kidneys, ureters, bladder and urethra. Bacteriuria can lead to the development of cystitis or pyelonephritis.

In this study, a total of 252 urine specimens from suspected UTI patients were collected from DHQ hospital Kohat and Hangu and from CMH Kohat and Hangu (Thall). Specimen reactive to nitrate strip reduction test were inoculated on CLED media, incubated at 37°C for 24 hours. 175 (69.44%) urine was positive for nitrate reduction test, included 135 (77.14%) *E. coli*, 33 (18.85%) *Klebsiella* and 7(4.0%) *Proteus,* verified through biochemical tests.

E. coli was found to be the most frequent (78%) isolates in UTI patients in our research work which in accordance with the previous studies.[2, 16] Furthermore, our research results are in close proximity with A. Fawwad el at.[17] The isolates *E. coli, Klebsiella and Proteus* were screened for their sensitivity pattern against different fluoroquinolone group of antibi-

otics such as ciprofloxacin, levofloxacin, norfloxacin, ofloxacin, moxifloxacin. The results showed that 51 (37.22%) out of 135 *E. coli*, 9 (27.27%) out of 33 *Klebsiella* and 2 (28.57%) out of 7 *Proteus* were resistant to most fluoroquinolones. Our results are in accordance with figures observed by A Rehman et al reporting 32.5% *E. coli* resistance to ciprofloxacin and 34.4% resistance to norfloxacin.[18] Grillon et al. reported that 75% *Klebsiella* were susceptible to both ciprofloxacin and levofloxacin and 67.5% to moxifloxacin. These findings are in contrast to ours showing 72.73% *Klebsiella* susceptible to both ciprofloxacin and levofloxacin while 81.82% to moxifloxacin. This is because that ciprofloxacin and levofloxacin advise frequently both in districts kohat and hangu thus organisms acquire resistant mechanism against the use such antibiotics while moxifloxacin is rarely advised in both district that's why showed more sensitivity.[19] In resistant fluoroquinolones antibiotics, moxifloxacin was the least resistant of all to nitrate reducing bacterial isoltaes as it is an updated fourth generation fluoroquinolones that have high potency and less resistant isolates in community. Ofloxacin was the most resistant antibiotic as it is the second-generation antibiotic having less potency and bacteria are too much exposed to them, thus have acquired resistance mechanism. Levinson et al. reported that bacteria acquire mutation either in gyrA or gyrB genes become resistant to fluoroquinolones group of antibiotics.[7] Thus DNA gyrase encoding these genes were detected in our research study in nitrate reducing bacteria that were resistant to fluoroquinolones group of antibiotics.

CONCLUSIONS

The study concluded that the prevalence of *E. coli* was high in nitrate reducing uropathogens in Hangu and Kohat districts. Bacterial isolates obtained from Hangu district were more resistant to fluoroquinolones antibiotics as compared to bacterial isolates of Kohat district. The nitrate reducing uropathogens were mostly sensitive to moxifloxacin while resistant to ofloxacin. However, further sanger sequencing should be performed for the resistant nitrate reducing bacteria in order to read the sequence of gyrA and gyrB genes to find out the responsible mutation(s) for resistance.

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Conflict of Interest:

There is no conflict of interest in this study.

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Informed Consent Statement:

Informed consent was obtained from all subjects involved in the study.

Author Contributions:

Conceptualization, Amhad Ijaz and Hussain Mubashir.; Methodology: Nabeeb Nasir, Fozia Fozia.; Software: Nabeeb Nasir, Fozia Fozia.; Validation: Nabeeb Nasir, Amhad Ijaz and Hussain Mubashir.; Formal analysis: Ahmad Mushtaq, Ahmad Nisar.; Investigation: Nabeeb Nasir, Wahab Abdul.; Re-sources: Amhad Ijaz , UlHaq Zia.; Writing—review and editing: Nabeeb Nasir, Fozia Fozia. Hussain Mubashir; Supervision: Hussain Mubashir, Wahab Abdul.; Funding acquisition: Amhad Ijaz, UlHaq Zia; Amal Alotaibi.; Writing—review and editing. All authors have read and agreed to the published version of the manuscript.

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Figure 1: Nitrate reduction strip showing neagtive nitrate test.

Figure 2: Nitrate reduction strip test (Positive).

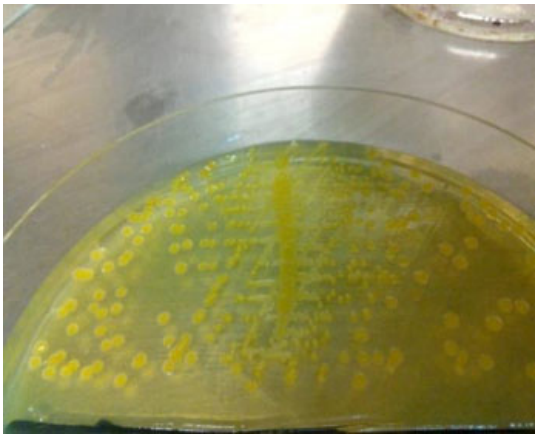

Figure 3: Culture of *E. coli.*

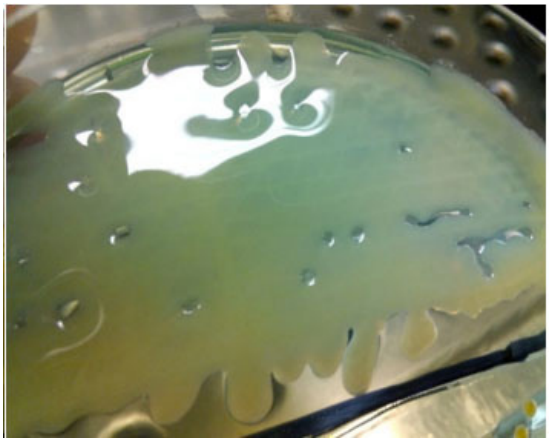

Figure 4: Culture of *Klebsiella.*

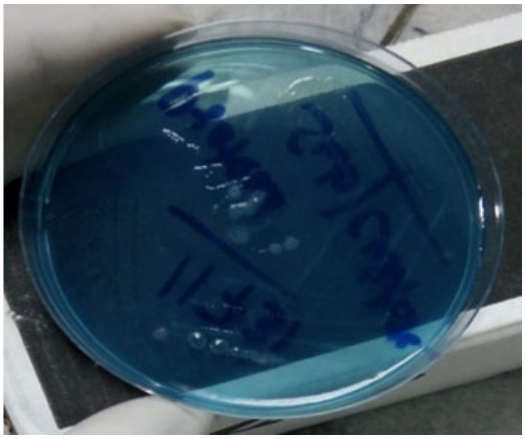

Figure 4: Culture of *Proteus.*

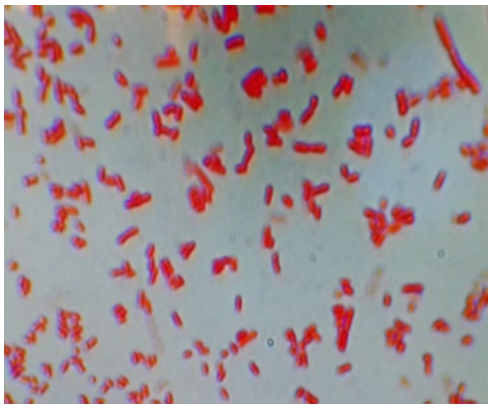

Figure 6: Gram stain.

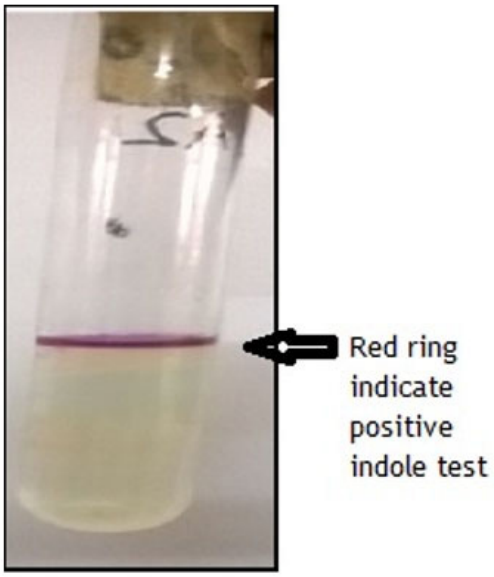

Figure 7: *Indole test for E. coli.*

Figure 8: An agar plate showing *E. coli* discsshowing resistance to five types of fluoroquinolone antibiotics. The discs placed were of moxifloxin, ofloxacin, ciprofloxacin, levofloxacin and norfloxacin. ETP and TZP both are control.

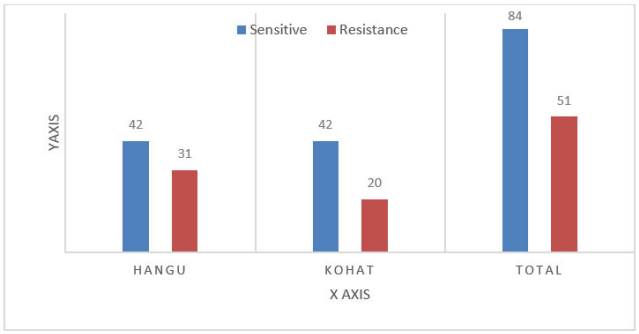

Figure 9: Antimicrobial sensitivity of *E. coli* to fluoroquinolones in districts Hangu and Kohat.

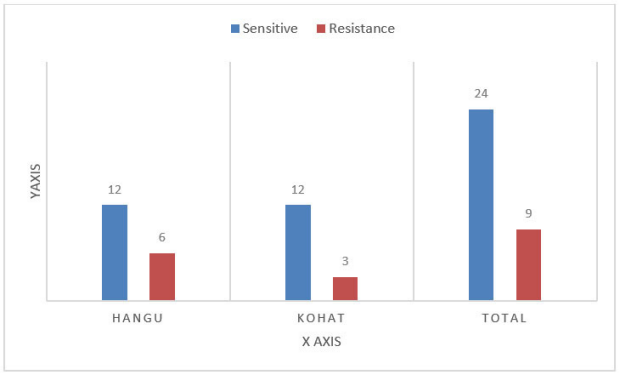

Figure 10: Antimicrobial sensitivity of *Klebsiella* to fluoroquinolonesin districts Hangu and Kohat.

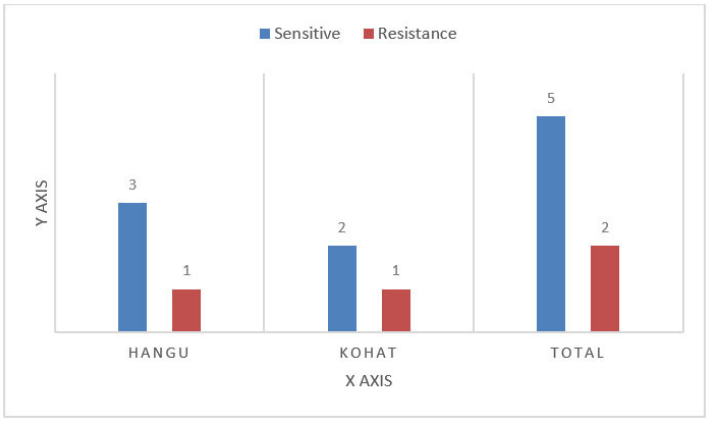

Figure 11: Antimicrobial sensitivity of *Proteus* in districts Hangu and Kohat.

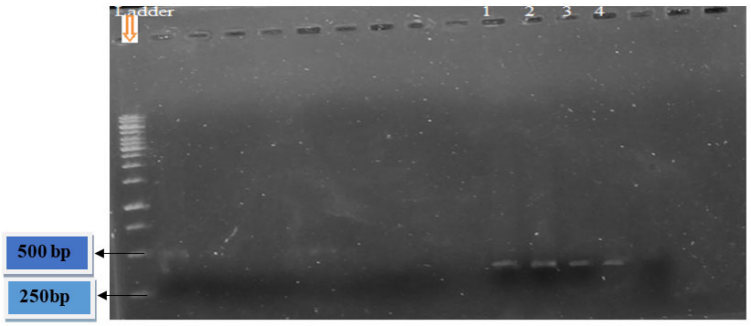

Figure 12: PCR products of gyrA gene of bacterial isolates *E. coli* (1, 2), *Klebsiella* (3) and (4) *Proteus*.

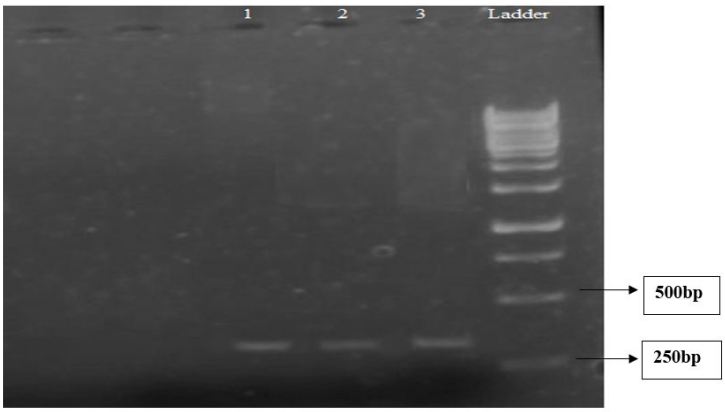

Figure 13: PCR products of gyrB gene of bacterial isolates *E. coli*(1),*Klebsiella*(2) and *Proteus* (3).

Table 1: Optimized conditions for amplification of gyrA and gyrB genes

Steps	Temperature (°C)	Time (Min)	No. of cycles
Initial Denaturation	94	2	1
Denaturation	94	1	35
Annealing	60	1	
Extension	72	1	
Final extension	72	5	1

Table 2: Frequency of nitrate reducing bacteria in both districts Hangu and Kohat

Total no. of suspected UTI specimens	Positive for nitrate reduction (%)	*E. coli* (%)	*Klebsiella* (%)	*Proteus* (%)
252	175 (69.44 %)	135 (78%)	33 (18%)	7 (4 %)

Table 3: Frequency of nitrate reducing bacteria in district Hangu

Total no. of suspected UTI specimens	Positive for nitrate reduction (%)	*E. coli* (%)	*Klebsiella* (%)	*Proteus* (%)
126	95 (75.39%)	73 (76.8 %)	18 (18.9 %)	4 (4.21 %)

Table 4: Frequency of nitrate reducing bacteria in district Kohat

Total no. of suspected UTI specimens	Positive for nitrate reduction (%)	*E. coli* (%)	*Klebsiella* (%)	*Proteus* (%)
126	80 (63.49%)	62 (77.5%)	15 (18.7 %)	3 (3.75%)

Table 5: Antibiotic sensitivity *E. coli* isolates from Kohat & Hangu districts

S. No	Tested Antibiotics	Abbreviation	Susceptibility (Percentage)	Resistance (Percentage)
1.	Ciprofloxicin	CIP	03 (5.88%)	48 (94.11%)
2.	Levofloxacin	LEV	02 (3.92 %)	49 (96.07%)
3.	Norfloxacin	NOR	03 (5.88%)	48 (94.11%)
4.	Ofloxacin	OFL	0 (0%)	51 (100%)
5.	Moxifloxacin	MXF	09 (17.64%)	42 (82.35%)

Table 6: Antibiotic sensitivity of *Klebsiella* isolates from Kohat & Hangu district

S. No	Tested Antibiotics	Abbreviation	Susceptibility (Percentage)	Resistance (Percentage)
1.	Ciprofloxicin	CIP	0.0 (0.00%)	09 (100.0%)
2.	Levofloxacin	LEV	0.0 (0.00%)	09 (100.0%)
3.	Norfloxacin	NOR	01 (11.11%)	08 (88.88%)
4.	Ofloxacin	OFL	0 (0%)	09 (100%)
5.	Moxifloxacin	MXF	03 (33.33%)	06 (66.66%)

Table 7: Antibiotic sensitivity of *Proteus* isolates from Kohat & Hangu district

S. No.	Tested Antibiotics	Abbreviation	Susceptibility (Percentage)	Resistance (Percentage)
1.	Ciprofloxicin	CIP	0.0 (0.0%)	02 (100%)
2.	Levofloxacin	LEV	0.0 (0.0%)	02 (100%)
3.	Norloxacin	NOR	0.0 (0.0%)	02 (100%)
4.	Ofloxacin	OFL	0.0 (0.0%)	02 (100%)
5.	Moxifloxacin	MXF	0.0 (0.0%)	02 (100%)