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BIOCHEMICAL CHARACTERIZATION AND ANTIBIOTIC SENSITIVITY PROFILING OF UROPATHOGENIC BACTERIAL STRAINS ISOLATED FROM URINE SAMPLE OF UTI PATIENTS

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ABSTRACT

Prevalence of Urinary tract infections (UTIs) holds the second position among the various infectious disease caused by uropathogenes. Abuse and improper prescribing policy of antibiotics induces multidrug resistance property among the causative agents of Urinary Tract Infections (UTIs).The main intend of this study was to isolate, biochemically characterize and to monitor the antibiotic susceptibility property among uropathogenic bacterial strains from urine samples of UTIs positive outdoor patients. A total number of twenty five (25) UTI patient's left over urine samples were collected from the various pathological laboratories at Malda Town among them twenty two (22) gave significant culture growth responses. Fifty two (52) bacterial strains were isolated from the twenty two (22) urine samples, whereas 42.30% were gram positive and 57.69% were gram negative strains. Characterization of isolated strains was determined by using different selective media and various biochemical assays such as, IMViC, Oxidase, gelatine hydrolysis, motility and urease assays etc. The maximum numbers of isolated bacterial strains were *Escherichia coli* and *Staphylococcus aureus*. In this study, 83.33% of gram negative bacteria were indole positive and 96% of indole positive bacterial isolates were MR positive and 100% of indole positive bacterial strains were VP negative. Entire uropathogenic isolates were showed the positive results in the nitrate reduction assay. Antibiotic sensitivity profile suggested that, all the isolated strains were multi-drug resistant, among them a very few isolates were sensitive of the applied antibiotics. Thus, a strict regulatory approach should be applied to control such massive spread of antibiotic resistance.

Keywords: Urinary Tract Infections (UTIs), Uropathogenic Bacteria, Antibiotic Sensitivity Pattern

1. INTRODUCTION

Among the globally infectious disease which enhance the socio economic encumber on the public health, urinary tract infection is the familiar one [1]. Urinary tract infection (UTI) is a common inflammatory disorder of the urinary system causes by the uncontrolled growth of the microorganism. UTIs are the one of the most dangerous nosocomial infection occurring specifically in women. At least once in their lifetime nearly 50-60% of women's are suffering from urinary tract infections in worldwide [2]. As reported by various scientists and clinicians, UTIs are responsible for the short term anguish in form of lower abdominal pain, headache, fever and dysuria, which's may be cause to permanent damage of kidney [3-4]. UTIs may be asymptomatic, symptomatic, acute and complicated or uncomplicated but both symptomatic and asymptomatic urinary tract infection responsible for the major illness [5]. Gram positive and gram negative both type of bacteria and some fungus can be responsible for the UTIs. The chief prevailing microorganism is Uropathogenic *Escherichia coli* (UPEC). The Gram-positive bacteria include *Staphylococcus sp, Streptococcus sp and Enterococcus sp. and* gram-negative includes a large number of aerobic bacilli such as *Klebsiella sp, Enterobacter sp, Citrobacter sp, Proteus sp, Serratia sp, Salmonella sp and Pseudomonas sp* [6-9]. Among these microorganisms *Klebsiella pneumoniae, Proteus mirabilis, Staphylococcus aureus, Enterococcus faecalis* are the most frequently isolated but 80-90% of infections are caused by the gram negative UPEC [10-12]. Normally UTIs is treated by the broad spectrum of antibiotics. The over prescription and use of antibiotics without performing culture and proper testing may contribute to the severe increase in antibiotic resistance [13-14]. The World Health Organization newly highlighted the increased incidence of *Escherichia coli* resistant to fluoroquinolones and third generation cephalosporins.

Extended-spectrum β-lactamase (ESBL) producing Enterobacteriaceae have also been identified as a serious threat by the Centre for Disease Control and Prevention [15]. Inapt use of antibiotics creates an alarming situation globally as well as plays a critical role in research field. Previous few decades science researcher cannot able to discover any type of antibiotics against the resistance bacteria [16-17], which leading to the emergence of multidrug resistance strains of pathogenic bacteria [18]. The World Health Organization (WHO) take a foot stapes against this alarming condition, launched the global National Action Plan (NAP) program on antibiotic resistance in 2015 [19]. In these present circumstances, prolonged use of antibiotics is changing the resistance pattern through plasmids and drastic mutation process and the new MDR bacteria are recurrently boost their morbidity and mortality [20]. Our aim of this study is to isolate, characterize and identify the UTI causing bacteria from the urine sample of the UTI positive patients and also to evaluate the pattern of antibiotic resistance which might be applied as appropriate guidelines for the future usage of antibiotics to treat UTIs.

2. MATERIAL AND METHODS

2.1. Material

2.1.1. Sample collection

Urine sample was collected from the UTI infected patients in various pathological laboratories at Malda town, Dist- Malda, West Bengal, India. The Urine samples were primarily tested and confirmed by the lab technician. Only primary screened UTI positive left over urine samples were brought to our laboratory and were further cultured and processed for several experiments. The study design has been approved by Institutional Ethics Committee (Human) of University of Gour Banga, Malda (Approval no: UGB/IEC (Human)/004-19).

2.1.2. Culture media and Chemicals

To full fill this study different kind are culture media and chemical have used including Luria Broth, Nutrient Broth, MR-VP Broth Medium, Tryptone Soya Broth, Nutrient Agar, Manitol Salt Agar Base, TITG Agar Base (Enterococcus Differential Agar), Dnase Test Agar W/ Methyl Green, Triple Sugar Iron (TSI) Agar, Gram's Ctystal Violet, Gram's iodine, Gram's Safranin,0.5% w/v, Gram's Decolourizer, Ethyl Alcohol, Kovac's reagent, Methyl red(2-(N,N-dimethyl-4-aminophenyl) azobenzenecarboxylic acid), α-Napthol, Simmons Citrate Agar, were purchased from Himedia, India, TriSodium citrate ($Na_3C_6H_5O_7$), hydrogen peroxide (H_2O_2), sodium hydroxide (NaOH), potassium chloride (KCl), sodium chloride (NaCl), Phosphate Buffer Saline (PBS), potassium hydroxide (KOH) were procured from Merck Ltd., SRL Pvt. Ltd., Mumbai, India. All Other Chemicals Were From Merck Ltd., SRL Pvt., Ltd., Mumbai and were of the highest grade available.

2.2. Methods

2.2.1. Colony identification and microscopic analysis

2.2.1.1. Bacterial Colony formation assay

Collected 22 urine samples were cultured into the nutrient broth then the broth cultured was inoculated into the nutrient agar plate as per Tanaka *et al.*, 2015 methods with some modification [21]. The plates were incubated into the 37°C incubator over night. After that visible different characteristic colonies were picked and sub-cultured into the fresh nutrient agar plate.

2.2.1.2. Bacterial Gram staining assay

After the colony formation assay, isolates were processed for the Gram staining process. Gram staining assay was performed according to standard method of Duguid JP, 1996 [22]. Slides were examined under phase contrast microscope and Gram positive and negative isolates were separated.

2.2.1.3. Potassium hydroxide (KOH) assay

To recheck Gram negative and Gram positive organisms KOH assay was performed. At first Bacterial culture was prepared on the nutrient agar plate. 10 µl of 3% potassium hydroxide solution was put on a new clean glass slide and one full loop bacterial culture was stir continuously for 60 sec and gently pulls the loop away from the suspension and observed for the changes [23].

2.2.2. Biochemical characteristic analysis

Initially selected picked colonies were separated and recognized according to the colony morphology and microscopic analysis through the gram staining assay. For the final confirmation of uropathogens, colonies were further examined using the various established biochemical tests, such as; IMViC assay (for gram negative only), Oxidase assay [24], Gelatine hydrolysis assay [25], Motility assay [26], Urease assay, Nitrate reduction assay and Catalase assay [27].

2.2.3. Screening through prepared media

Various culture media were used for the determination of uropathogenic bacterial strains. To examined the characteristics of obtained bacterial isolates the following assays were perforemed; Triple Sugar Iron (TSI),

Enterococcus differential agar base (TITG Agar Base), Mannitol salt agar, Blood agar assay and MacConkey agar assay. The entire mentioned agar mediums were autoclaved at 121°C for 15 min. [27].

2.2.4. Antibiotic susceptibility profile analysis

Antibiotic susceptibility test was performed using the Kirby-Bauer disk agar diffusion method described by Clinical Laboratory Standards Institute (CLSI, 2015) with some modification [28]. The antibiotic sensitivity profile of clinically isolates bacterial strains were demonstrated by using nine specific antibiotics [(Amikacin (30µg), Ciprofloxacin (5µg), Meropenem (10µg), Imipenem (10µg), Ceftazidime (30µg), Gentamycin (10µg), Tetracyclin (30µg), Cefixime (5µg) and Erthromycin (10µg)]. Isolated bacterial strains were culture in nutrient broth and incubated at 37°C for 18-24 hours. After the growing phase bacterial culture were again re-cultured according to the range of Mcfarland standards (0.5). These new bacterial culture was inoculated into the Mueller Hinton agar by the sterile swab stick. Plates were incubated at room temperature for 5-10 minutes and antibiotic discs were placed properly into the agar plate.

3. RESULTS AND DISCUSSION

3.1. Bacterial colony formation assay

A total of 25 UTI positive patients's left over urine samples were collected from the various pathological laboratories at Malda Town, West Bengal. Only 22 urine samples showed significant growth in colony formation assay. Only visible different bacterial colonies were collected for the further isolation and characterization process.

3.2. Bacterial gram staining assay

According to the different morphology a total number of 52 bacterial isolates were picked and separately cultured into the Nutrient broth medium. From the Gram staining assay, it was observed that, 57.69% isolates revealed characteristics of Gram negative bacterial strain and 42.30% isolates were carried the phenomenon of gram positive bacterial strain. These notable percentage of both (gram positive and gram negative) uropathogenic bacterial strains showed quite similar results compared to the previous study of Patra *et al.*, 2019 [29]. Clinically isolated bacterial strains were gram positive, which may be due to the having thick peptidoglycan layer with numerous teichoic acid cross-linking which resists the decolorization and crystal violet dissociates into CV+ and Cl- ions that penetrate through the wall and membrane. The CV+ interacts with the negatively charged components of bacterial cell, which presented as purple colour. In case of gram negative bacterial isolates Crystal-Violet-Iodine complex binds to the inner wall as well as into the outer wall. Interestingly outer layer losses its integrity and inner membrane become exposed during decolorization process, this may be due to the presence of membrane with thin peptidoglycan layer [30]. From the previous study it was well established that, gram negative uropathogens were the main causative agent for UTIs and very small amount of gram positive bacteria contributed for such complications [31-32], but our study significantly indicated that UTIs causing bacterial percentage were shifted towards the gram positive bacteria; this observation is highly correlated with the similar study of Sharma *et al.,* 2019 [28].

3.3. Potassium hydroxide (KOH) assay

Potassium hydroxide (KOH) assay was conducted for the rapid detection of bacterial isolates in a mix culture. Furthermore it is a confirmation tests for Gram staining assay. Total number of gram positive isolates showed the KOH negative result and entire number of gram negative bacterial strains gives KOH positive results *(Figure 1, Figure 2 and Table 1, Table 2).* Potassium hydroxide (KOH) assay obtain the same results, which were observed in bacterial gram staining assay.

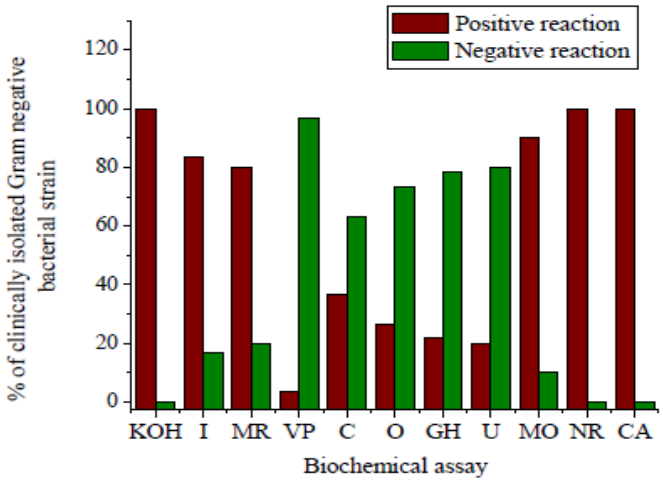

Fig. 1: Biochemical assay of clinically isolated gram negative bacterial strains. Here *(KOH): Potassium hydroxide assay, (I): Indole assay, (MR): Methyl Red assay, (VP): Voges-Proskauer assay, (C): Citrate Utilization assay, (O): Oxidase assay, (GH): Gelatine hydrolysis assay, (U): Urease test, (MO): Motility Assay, (NR): Nitrate reduction assay, (CA): Catalase test.*

3.4. IMViC assay

For the authentic identification and characterization of clinically isolated bacterial strains IMViC (Indole, Methyl red, Voges-Proskauer, and Citrate) tests were frequently performed but 42.30% of gram positive bacterial isolates were exempted for IMViC test. This study signify that 83.33% of clinically isolated gram negative bacterial strains were indole positive and 16.67% shows the negative reaction *(Figure 1 & Table 2)*. Among the gram negative bacterial isolates indole positive strains were can easily differentiate from most *Klebsiella sp and Enterobacter sp* [33]. Significantly Indole assay determine the potency of the indole positive isolates to break down the amino acid tryptophan into indole by the production of tryptophanase enzyme. Final products (Indole) of this reaction are generated by the reductive deamination from tryptophan due to the presence of intermediate molecule indolepyruvic acid and tryptophanase enzyme catalyzes the reaction, which ends with the removal of tryptophan molecule [33-34]. Amid the gram negative isolates 96% of indole positive bacterial isolates were MR positive and 100% of indole positive bacterial strains were VP negative *(Figure 1 and Table 2)*. From MR positive isolates, they have the potency to utilise glucose with the production of a stable acid. Quite similarly VP negative bacterial isolates are enabling to produces acetylmethyl carbinol from glucose fermentation [35]. According to this study, *Figure 2 and Table 2* indicates the all the indole positive bacterial isolates showed negative results in citrate utilization assay. Previous science report [36], it was established that non metabolism of citrate compound is basic source of carbon in the media. As a result, under the normal state bromothymol blue did not able to change the media colour from green to blue. From the previous study of, it was established that, among the UTIs isolates maximum number of bacterial strains were *Escherichia coli* [27]. In the present study whereas indole positivity, MR positivity, VP negativity and Citrate negativity clearly demonstrated the presence of maximum number of *Escherichia coli* strains among the clinical isolates *(Table 2)*. Thus our study is highly correlates to the study of Dash *et al.*, 2012; Sharma *et al.,* 2019; Ahmed *et al.*, 2019 [27-28,37]. In the case of strain number MLD 41 indole positivity, MR negativity, VP positivity and citrate positivity notably indicates burly proof on behalf of *Klebsiella oxytoca*.

3.5. Oxidase assay

Oxidase test was conduct in this study to determine bacteria that produce Cytochrome c oxidase, an enzyme which part of their respiratory chain. Around 81% of gram positive bacterial isolates can able to produce cytochrome c oxidase, which may oxidizes the reagent (tetramethyl-p-phenylenediamine) to (indophenols) purple colour end product *(Figure 2 and Table 1)*. But 73.33% of gram negative isolates indicates oxidase negative reaction, due to the lacking of cytochrome c as the part of their electron transport chain and therefore do not oxidize the reagent *(Figure 1 and Table 2)*. Significantly oxidase negative results showed strong evidence for the *Enterobacteriaeae* family [38].

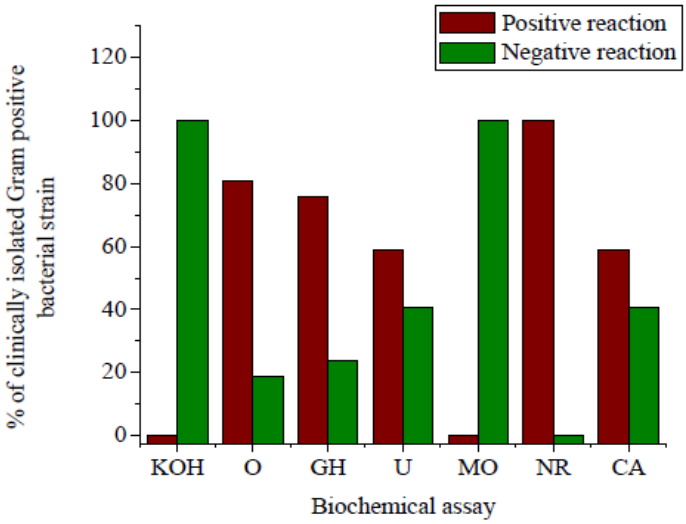

Fig. 2: Biochemical assay of clinically isolated gram positive bacterial strains. Here *(KOH): Potassium hydroxide assay, (O): Oxidase assay, (GH): Gelatine hydrolysis assay, (U): Urease test, (MO): Motility Assay, (NR): Nitrate reduction assay, (CA): Catalase test.*

3.6. Gelatine hydrolysis assay

Around 78.12% of gram negative bacterial isolates were showing negative results *(Figure 1& Table 2)*, where as 76 % of gram positive isolated strains presented the positive result *(Figure 2 & Table 1)*. Positive reaction of gelatin hydrolysis assay indicated the production of gelatinase enzyme, which needed for the breakdown of gelatin [39]. Interestingly few bacterial isolates gave erratic (Positive or Negative) results, those strains (MLD 1,MLD 20, MLD 25, MLD 30 and MLD 47) were not incorporated in this assay *(Table 1)*.

3.7. Urease assay

Urease assay was performed for the characterization of urea hydrolysis capability of urinary tract isolates. Among the gram positive bacteria 59.09% of bacterial strains were urease positive and 40.91% of strains showed negative results *(Figure 2 & Table 1)*. Most of the gram negative uropathogens signified the urease negative results (80%), this may be due to the absence of the urease enzyme essential for hydrolysis of urea to ammonia [33]. Considerably strain number MLD 3, MLD 17, MLD 28, MLD 45 and MLD 48 gives the variable results against this biochemical assay, so this isolates were not incorporated *(Figure 1 and Table 2)*.

3.8. Triple sugar Iron (TSI) assay

Triple Sugar Iron agar was used for the determination of gram negative *Enterobacteriaceae* on the basis of hydrogen sulphide production and dextrose, lactose and sucrose fermentation according to the Indian pharmacopoeia (1996). It was observed in this study that, 90% of gram negative isolates showed a positive result except MLD 13, MLD 35 and MLD 39 strains *(Table 2)*.

3.9. Enterococcus differential agar base (TITG Agar Base)

TITG agar medium is a selective differential agar base for the detection of *Enterococci* bacteria from the bacterial mixed culture [40]. Especially TITG agar was used in this assay to differentiate *Enterococcus faecalis* and *Enterococcus faecium* from the various uropathogenic gram positive bacterial isolates. This differentiation is based on the reduction of tetrazolium, so the *Enterococcus faecalis* shows deep red color colonies whereas *Enterococcus faecium* produces colorless colonies. As per the result strains number MLD 1, MLD 20, MLD 25, MLD 30 and MLD 47 are strongly support the evidence on behalf of *Enterococcus faecalis (Table 1)* and strain number MLD7, MLD 12, MLD 37 and MLD 50 is *Enterococcus faecium (Table 1)*.

3.10. Motility assay

So 100% of gram positive bacterial strain gives the negative results in this biochemical assay *(Figure 2 & Table 1)*. Major number (90%) of gram negative isolates were motile *(Figure 1)* except MLD 5 and MLD 46 strains these strains were found non motile during this assay *(Table 2)*.

3.11. Nitrate reduction assay

Notably all of the bacterial isolates (100%) showed the positive results during this experiment *(Figure 1 & Figure 2)*.

3.12. Catalase test

In this assay, it was found that 86.53% of bacterial isolates were catalase positive. Significantly entire gram negative bacterial strains were gives catalase positive results *(Figure 1 & Table 2)*, whereas among the gram positive isolates 40.91% strains were showed catalase negative results *(Figure 2 & Table 1)*.

3.13. Mannitol salt agar and MacConkey agar assay

The entire gram negative bacterial isolates cultures growths were inhibited in the Mannitol salt agar medium *(Table 2),* whereas total gram positive isolates (100%) indicated the positive results in this assay *(Table 1).* Significantly entire gram positive bacterial strains (100%) were showed the negative results and gram negative bacterial strains produced different color colonies. As per the results, it was confirmed that MLD 4, MLD 10, MLD 21, MLD 24, MLD 32, MLD 40, MLD 43 and MLD 51 strains are the *Staphylococcus aureus* and MLD 9, MLD 18, MLD 27 and MLD 34 are the *Staphylococcus epidermidis.* Among the gram positive bacterial strains 40.9% of isolates are *Staphylococcus aureus,* 22.7% are *Enterococcus faecalis,* 18.2% are *Enterococcus faecium* and 18.2% isolates are *Staphylococcus epidermidis (Figure 4)*.

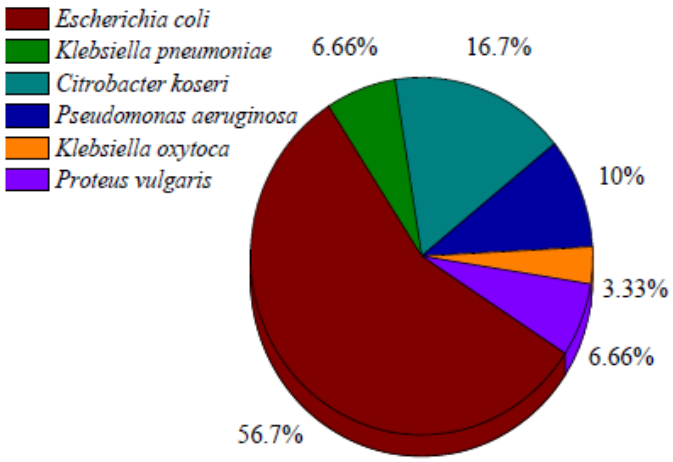

Fig. 3: Percentage of isolated Gram negative bacterial strains from the left over sample of UTIs

3.14. Antibiotic susceptibility testing

A subset of microbial cells derived from a vulnerable population that may able to develop mutations in genes that affect the activity of the antimicrobial agents, which leads to a preserved cell survival against the recent antibiotics [41]. Antibiotic susceptibility profiles of clinically isolated bacterial strains were observed against the nine (9) antimicrobial drugs that are presented in

Figure 5 and Figure 6. Multiple resistances were found majorly among the isolated gram negative strains. In our study *E.coli* was the main UTI causing bacteria *(Figure 3);* the others study reports across the India revealed the same scenario [42-44]. High amount of *E.coli* (86%) isolates were found in study conducted by Majumder *et al*. in Bangladesh compared to 56.66 % seen in our study [45]. From the antibiotic susceptibility of assay E.coli showed highest 88.23 % sensitivity against Meropenem (10µg) and 82.35% against Imipenem (10 µg); this results strongly correlates with the various science report across the India [46-47].

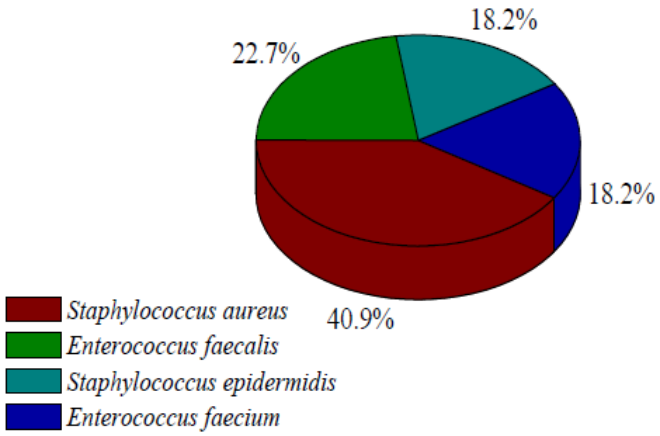

Fig. 4: Percentage of isolated Gram Positive bacterial strains from the left over sample of UTI patients.

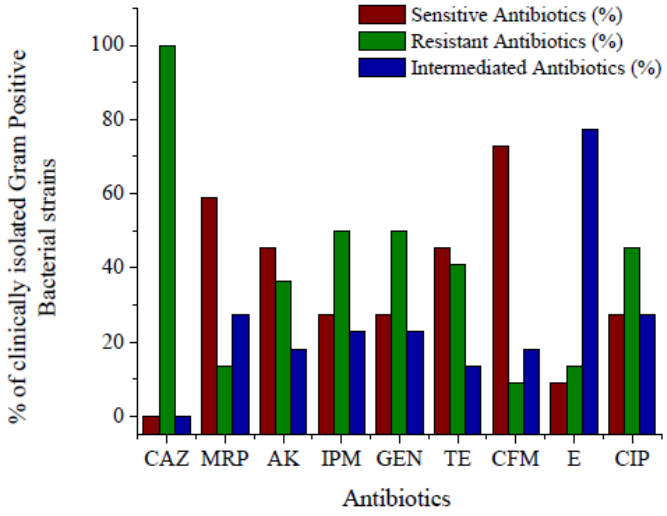

Fig. 5: Graphical representation of isolated gram positive Uropathogens against the various antimicrobial drugs. Here, ***(CAZ): Ceftazidime 30µg, (MRP): Meropenem 10µg, (AK): Amikacin 30µ, (IPM): Imipenem 10µg, (GEN): Gentamycin 10µg, (TE): Tetracyclin 30µg, (CFM): Cefixime 5µg, (E): Erythromycin 10µg, (CIP): Ciprofloxacin 5µg***

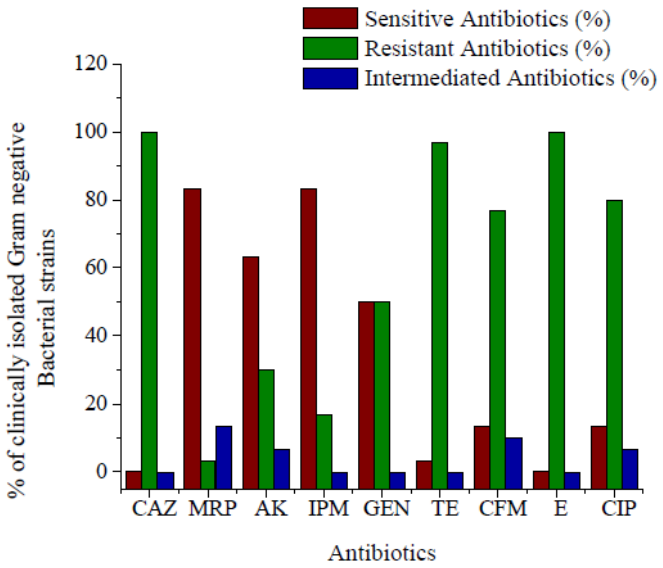

Fig. 6: Graphical representation of isolated gram negative Uropathogens against the various antimicrobial drugs. Here, ***(CAZ): Ceftazidime 30µg, (MRP): Meropenem 10µg, (AK): Amikacin 30µ, (IPM): Imipenem 10µg, (GEN): Gentamycin 10µg, (TE): Tetracyclin 30µg, (CFM): Cefixime 5µg, (E): Erythromycin 10µg, (CIP): Ciprofloxacin 5µg***

Interestingly, a previous study in Lahore, Pakistan opposes this out comes through the very low sensitivity profile 39.5% against Imipenem (10 µg) [48]. In this study, 100% of isolated *E.coli* (MLD 2, MLD 6, MLD 8, MLD 14, MLD 16, MLD 19, MLD 22, MLD 23, MLD 26, MLD 29, MLD 31, MLD 33, MLD 38, MLD 42, MLD 44, MLD 49, MLD 52) were resistance to Ceftazidime (30µg), Cefixime (5µg), Erthromycin (10µg) and Ciprofloxacin (5µg); 94.18 % of isolated *E.coli* (MLD 2, MLD 6, MLD 8, MLD 14, MLD 16, MLD 19, MLD 22, MLD 23, MLD 26, MLD 29, MLD 31, MLD 33, MLD 42, MLD 44, MLD 49, MLD 52) were resistance to Tetracyclin (30µg); 47.06% of *E.coli* (MLD 8, MLD 16, MLD 19, MLD 23, MLD 31, MLD 33, MLD 42, MLD 49) were resistant to Gentamycin (10µg); 41.82 % of uropathogenic *E.coli* (MLD 2, MLD 16, MLD 19, MLD 31, MLD 33, MLD 42, MLD 49) were resistant against to Amikacin (30µg). In case of Imipenem (10 µg) MLD 2, MLD 31 and MLD 49 were showed resistance and Meropenem (10µg) showed resistance to MLD 49 as well as partial inhibition to the strain number MLD 16. Among the gram negative bacterial strains it was indicated that, high resistance property to the six (6) antibiotic found in the following descending order: Ceftazidime (30µg), Erthromycin (10µg), Tetracyclin (30µg), Cefixime (5µg), and

Gentamycin (10µg) and Ciprofloxacin (5µg) *(Figure 6).* According to the antibiotic resistance *Klebsiella spp* also showed high resistant against the various antibiotics. Entire (100%) isolated *Klebsiella spp* (MLD 5, MLD 41, MLD 46) were resistant to Ceftazidime (30µg), Gentamycin (10µg), Tetracyclin (30µg), Cefixime (5µg), Erythromycin (10µg) and Ciprofloxacin (5µg); this observation nearly similar to the study of George *et al*., India [49]. *Citrobacter koseri* is the second most isolated bacterial strains among the gram negative isolates; interestingly Meropenem (10µg), Imipenem (10 µg) and Gentamycin (10µg) achieved the complete sensitivity against the entire isolated *Citrobacter koseri (*MLD 3, MLD 17, MLD 28, MLD 45 and MLD 48). However, in our study uropathogenic gram positive bacterial isolates were gave better sensitivity profile against the antibiotics. A total of 72.72 % gram positive bacterial isolates were sensitive to the Cefixime (5µg); whereas 59.09 % of sensitivity showed by the Meropenem (10µg) and only Ceftazidime (30µg) indicated the complete resistance to the entire gram positive strains *(Figure 5).* Strain number MLD 4, MLD 10, MLD 15, MLD 21, MLD 24, MLD 32, MLD 40, MLD 43 and MLD 51were partially inhibited to the Imipenem (10 µg) and Erthromycin (10µg); where as 33.33% of *Staphylococcus aureus (MLD 15, MLD 24, MLD 32)* were resistant to the Amikacin (30µg). 77.78% of isolated *Staphylococcus aureus* (MLD 10, MLD 15, MLD 21, MLD 24, and MLD 51) were sensitive to the Gentamycin (10µg). In this scenario it has been clearly understood that near about all the uropathogenic strains are multi-drug resistance. So, there is urgency for establishment a brand new antibiotic against the urinary tract infections isolated bacterial strains, which should be followed all the concerned authorities. This is may be the only way to control the antibiotic resistant property.

Table 1: Standard biochemical tests of uropathogenic Gram Positive clinical isolates, collected from urine sample of UTI patient.

Here, ***(KOH)*** = *Potassium hydroxide assay* ***(O)*** = Oxidase assay, ***(GH)*** = Gelatine hydrolysis assay, ***(U)*** = Urease assay, ***(TSI)*** = Triple Sugar Iron, ***(MO)*** = Motility observation, ***(NR)*** = Nitrate reduction, ***(CAT)*** = Catalase assay. ***ND*** =Tests are not done, ***(+)*** = Tests are positive, ***(-)*** = Tests are negative.

S.N	Strain No	Gram staining	KOH assay	O	GH	U	TSI assay	TITG agar assay	Mannitol agar	MO	NR	CAT	Blood agar	MacConkey agar assay	Bacteria name
1	MLD 1	+	–	–	–/+	–	+ S: Yellow B: Red G: No H_2S: No	Good-luxuriant growth (red color)	(+) Yellow colony is produced	Non-Motile	+	-–	ND	–	*Enterococcus faecalis*
2	MLD 4	+	–	–	+	+	+ S: Yellow B: Yellow G: No H_2S: No	No growth	(+) Yellow colony is produced	Non-Motile	+	+	Visible growth (Beta Hemolysis)	–	*Staphylococcus aureus*
3	MLD 7	+	–	+	+	–	+ S: Yellow B: Red G: No H_2S: No	Good-luxuriant growth (colorless)	(+) Yellow colony is produced	Non-Motile	+	–	ND	–	*Enterococcus faecium*
4	MLD 9	+	–	–	–	+	+ S: Yellow B: Yellow G: No H_2S: No	No growth	(+) Red colony is produced	Non-Motile	+	+	Visible growth (No Hemolysis)	–	*Staphylococcus epidermidis*
5	MLD 10	+	–	–	+	+	+ S: Yellow B: Yellow G: No H_2S: No	No growth	(+) Yellow colony is produced	Non-Motile	+	+	Visible growth (Beta Hemolysis)	–	*Staphylococcus aureus*
6	MLD 12	+	–	+	+	–	+ S: Yellow B: Red G: No H_2S: No	Good-luxuriant growth (colorless)	(+) Yellow colony is produced	Non-Motile	+	–	ND	–	*Enterococcus faecium*

7	MLD 15	+	–	–	+	+	+ S: Yellow B: Yellow G: No H_2S: No	No growth	(+) Yellow colony is produced	Non-Motile	+	+	Visible growth (Beta Hemolysis)	–	*Staphylococcus aureus*
8	MLD 18	+	–	–	–	+	+ S: Yellow B: Yellow G: No H_2S: No	No growth	(+) Red colony is produced	Non-Motile	+	+	Visible growth (No Hemolysis)	–	*Staphylococcus epidermidis*
9	MLD 20	+	–	–	–/+	–	+ S: Yellow B: Red G: No H_2S: No	Good-luxuriant growth (red color)	(+) Yellow colony is produced	Non-Motile	+	–	ND	–	*Enterococcus faecalis*
10	MLD 21	+	–	–	+	+	+ S: Yellow B: Yellow G: No H_2S: No	No growth	(+) Yellow colony is produced	Non-Motile	+	+	Visible growth (Beta Hemolysis)	–	*Staphylococcus aureus*
11	MLD 24	+	–	–	+	+	+ S: Yellow B: Yellow G: No H_2S: No	No growth	(+) Yellow colony is produced	Non-Motile	+	+	Visible growth (Beta Hemolysis)	–	*Staphylococcus aureus*
12	MLD 25	+	–	–	–/+	–	+ S: Yellow B: Red G: No H_2S: No	Good-luxuriant growth (red color)	(+) Yellow colony is produced	Non-Motile	+	–	ND	–	*Enterococcus faecalis*
13	MLD 27	+	–	–	–	+	+ S: Yellow B: Yellow G: No H_2S: No	No growth	(+) Red colony is produced	Non-Motile	+	+	Visible growth (No Hemolysis)	–	*Staphylococcus epidermidis*
14	MLD 30	+	–	–	–/+	–	+ S: Yellow B: Red G: No H_2S: No	Good-luxuriant growth (red color)	(+) Yellow colony is produced	Non-Motile	+	–	ND	–	*Enterococcus faecalis*

15	MLD 32	+	–	–	+	+	+ S: Yellow B: Yellow G: No H_2S: No	No growth	(+) Yellow colony is produced	Non-Motile	+	+	Visible growth (Beta Hemolysis)	–	*Staphylococcus aureus*
16	MLD 34	+	–	–	–	+	+ S: Yellow B: Yellow G: No H_2S: No	No growth	(+) Red colony is produced	Non-Motile	+	+	Visible growth (No Hemolysis)	–	*Staphylococcus epidermidis*
17	MLD 37	+	–	+	+	–	+ S: Yellow B: Red G: No H_2S: No	Good-luxuriant growth (colorless)	(+) Yellow colony is produced	Non-Motile	+	–	ND	–	*Enterococcus faecium*
18	MLD 40	+	–	–	+	+	+ S: Yellow B: Yellow G: No H_2S: No	No growth	(+) Yellow colony is produced	Non-Motile	+	+	Visible growth (Beta Hemolysis)	–	*Staphylococcus aureus*
19	MLD 43	+	–	–	+	+	+ S: Yellow B: Yellow G: No H_2S: No	No growth	(+) Yellow colony is produced	Non-Motile	+	+	Visible growth (Beta Hemolysis)	–	*Staphylococcus aureus*
20	MLD 47	+	–	–	–/+	–	+ S: Yellow B: Red G: No H_2S: No	Good-luxuriant growth (red color)	(+) Yellow colony is produced	Non-Motile	+	–	ND	–	*Enterococcus faecalis*
21	MLD 50	+	–	+	+	–	+ S: Yellow B: Red G: No H_2S: No	Good-luxuriant growth (colorless)	(+) Yellow colony is produced	Non-Motile	+	–	ND	–	*Enterococcus faecium*
22	MLD 51	+	–	–	+	+	+ S: Yellow B: Yellow G: No H_2S: No	No growth	(+) Yellow colony is produced	Non-Motile	+	+	Visible growth (Beta Hemolysis)	–	*Staphylococcus aureus*

Table 2: Standard biochemical tests of uropathogenic Gram Negative clinical isolates, collected from urine sample of UTI patient.
Here, ***(I)*** = Indole test, ***(MR)*** = Methyl Red test ***(VP)*** = Voges-Proskauer test, ***(C)*** = Citrate utilization test ***(O)*** = Oxidase assay, ***(GH)*** = Gelatine hydrolysis assay, ***(U)*** = Urease assay, ***(TSI)*** = Triple Sugar Iron, ***(MO)*** = Motility observation, ***(NR)*** = Nitrate reduction, ***(CAT)*** = Catalase assay.
ND =Tests are not done, ***(+)*** = Tests are positive, ***(-)*** = Tests are negative.

Sl. No	*Strain No*	*Gram stain*	*KOH Assay*	*I*	*MR*	*VP*	*C*	*O*	*GH*	*U*	*TSI*	*Mannitol agar*	*MO*	*Blood agar*	*NR*	*CAT*	*MacConkey agar*	*Bacteria Name*
1	MLD 2	–	+	+	+	–	–	–	–	–	+ S: Yellow B: Red G: Yes H_2S: No	(–) Inhibited	Motile	Visible growth (No hemolysis)	+	+	(+) Pink to red colony production	*Escherichia coli*
2	MLD 3	–	+	+	+	–	+	+	–	ND	+ S: Yellow B: Red G: Yes H_2S: Black	ND	Motile	Visible growth (Hemolysis)	+	+	(+) Pale colony production	*Citrobacter koseri*
3	MLD 5	–	+	–	–	+	+	–	–	+	+ S: Yellow B: yellow G: Yes H_2S: No	ND	Non-Motile	Visible growth (No hemolysis)	+	+	(+) Grey white colony production	*Klebsiella pneumoniae*
4	MLD 6	–	+	+	+	–	–	–	–	–	+ S: Yellow B: Red G: Yes H_2S: No	(–) Inhibited	Motile	Visible growth (No hemolysis)	+	+	(+) Pink to red colony production	*Escherichia coli*
5	MLD 8	–	+	+	+	–	–	–	–	–	+ S: Yellow B: Red G: Yes H_2S: No	(–) Inhibited	Motile	Visible growth (No hemolysis)	+	+	(+) Pink to red colony production	*Escherichia coli*
6	MLD 11	–	+	+	+	–	–	–	+	+	+ S: Red B: Yellow G: No H_2S: Black	(–) Inhibited	Motile	Visible growth (No hemolysis)	+	+	(+) Colorless colony production	*Proteus vulgaris*

7	MLD 13	–	+	–	–	–	+	+	+	–	– S: Red B: Red G: No H_2S: No	ND	Motile	Visible growth (Beta hemolysis)	+	+	(+) Pale white colony production	*Pseudomonas aeruginosa*
8	MLD 14	–	+	+	+	–	–	–	–	–	+ S: Yellow B: Red G: Yes H_2S: No	(–) Inhibited	Motile	Visible growth (No hemolysis)	+	+	(+) Pink to red colony production	*Escherichia coli*
9	MLD 16	–	+	+	+	–	–	–	–	–	+ S: Yellow B: Red G: Yes H_2S: No	(–) Inhibited	Motile	Visible growth (No hemolysis)	+	+	(+) Pink to red colony production	*Escherichia coli*
10	MLD 17	–	+	+	+	–	+	+	–	ND	+ S: Yellow B: Red G: Yes H_2S: Black	ND	Motile	Visible growth (Hemolysis)	+	+	(+) Pale colony production	*Citrobacter koseri*
11	MLD 19	–	+	+	+	–	–	–	–	–	+ S: Yellow B: Red G: Yes H_2S: No	(–) Inhibited	Motile	Visible growth (No hemolysis)	+	+	(+) Pink to red colony production	*Escherichia coli*
12	MLD 22	–	+	+	+	–	–	–	–	–	+ S: Yellow B: Red G: Yes H_2S: No	(–) Inhibited	Motile	Visible growth (No hemolysis)	+	+	(+) Pink to red colony production	*Escherichia coli*
13	MLD 23	–	+	+	+	–	–	–	–	–	+ S: Yellow B: Red G: Yes H_2S: No	(–) Inhibited	Motile	Visible growth (No hemolysis)	+	+	(+) Pink to red colony production	*Escherichia coli*
14	MLD 26	–	+	+	+	–	–	–	–	–	+ S: Yellow B: Red G: Yes H_2S: No	(–) Inhibited	Motile	Visible growth (No hemolysis)	+	+	(+) Pink to red colony production	*Escherichia coli*

15	MLD 28	–	+	+	+	–	+	+	–	ND	+ S: Yellow B: Red G: Yes H_2S: Black	ND	Motile	Visible growth (Hemolysis)	+	+	(+) Pale colony production	*Citrobacter koseri*
16	MLD 29	–	+	+	+	–	–	–	–	–	+ S: Yellow B: Red G: Yes H_2S: No	(–) Inhibited	Motile	Visible growth (No hemolysis)	+	+	(+) Pink to red colony production	*Escherichia coli*
17	MLD 31	–	+	+	+	–	–	–	–	–	+ S: Yellow B: Red G: Yes H_2S: No	(–) Inhibited	Motile	Visible growth (No hemolysis)	+	+	(+) Pink to red colony production	*Escherichia coli*
18	MLD 33	–	+	+	+	–	–	–	–	–	+ S: Yellow B: Red G: Yes H_2S: No	(–) Inhibited	Motile	Visible growth (No hemolysis)	+	+	(+) Pink to red colony production	*Escherichia coli*
19	MLD 35	–	+	–	–	–	+	+	+	–	– S: Red B: Red G: No H_2S: No	ND	Motile	Visible growth (Beta hemolysis)	+	+	(+) Pale white colony production	*Pseudomonas aeruginosa*
20	MLD 36	–	+	+	+	–	–	–	+	+	+ S: Red B: Yellow G: No H_2S: Black	(–) Inhibited	Motile	Visible growth (No hemolysis)	+	+	(+) Colorless colony production	*Proteus vulgaris*
21	MLD 38	–	+	+	+	–	–	–	–	–	+ S: Yellow B: Red G: Yes H_2S: No	(–) Inhibited	Motile	Visible growth (No hemolysis)	+	+	(+) Pink to red colony production	*Escherichia coli*
22	MLD 39	–	+	–	–	–	+	+	+	–	– S: Red B: Red G: No H_2S: No	ND	Motile	Visible growth (Beta hemolysis)	+	+	(+) Pale white colony production	*Pseudomonas aeruginosa*

23	MLD 41	–	+	+	–	+	+	–	–	+	+ S: Yellow B: yellow G: Yes H_2S: No	ND	Non-Motile	Visible growth (No hemolysis)	+	+	(+) Pink colony production	*Klebsiella oxytoca*
24	MLD 42	–	+	+	+	–	–	–	–	–	+ S: Yellow B: Red G: Yes H_2S: No	(–) Inhibited	Motile	Visible growth (No hemolysis)	+	+	(+) Pink to red colony production	*Escherichia coli*
25	MLD 44	–	+	+	+	–	–	–	–	–	+ S: Yellow B: Red G: Yes H_2S: No	(–) Inhibited	Motile	Visible growth (No hemolysis)	+	+	(+) Pink to red colony production	*Escherichia coli*
26	MLD 45	–	+	+	+	–	+	+	–	ND	+ S: Yellow B: Red G: Yes H_2S: Black	ND	Motile	Visible growth (Hemolysis)	+	+	(+) Pale colony production	*Citrobacter koseri*
27	MLD 46	–	+	–	–	+	+	–	–	+	+ S: Yellow B: yellow G: Yes H_2S: No	ND	Non-Motile	Visible growth (No hemolysis)	+	+	(+) Grey white colony production	*Klebsiella pneumoniae*
28	MLD 48	–	+	+	+	–	+	+	–	ND	+ S: Yellow B: Red G: Yes H_2S: Black	ND	Motile	Visible growth (Hemolysis)	+	+	(+) Pale colony production	*Citrobacter koseri*
29	MLD 49	–	+	+	+	–	–	–	–	–	+ S: Yellow B: Red G: Yes H_2S: No	(–) Inhibited	Motile	Visible growth (No hemolysis)	+	+	(+) Pink to red colony production	*Escherichia coli*
30	MLD 52	–	+	+	+	–	–	–	–	–	+ S: Yellow B: Red G: Yes H_2S: No	(–) Inhibited	Motile	Visible growth (No hemolysis)	+	+	(+) Pink to red colony production	*Escherichia coli*

4. CONCLUSION

From our study, it may concluded that fifty two (52) uropathogens were successfully isolated and identified from the UTIs patients left over urine sample by the use of traditional biochemical techniques. Among the entire isolates, highest numbers of uropathogenic *Escherichia coli (56.7%)* followed by *Staphylococcus aureus* (40.9%) strains were found. It is a massive alarming to note that Ceftazidime (30µg), Tetracyclin (30µg), Erythromycin (10µg), Ciprofloxacin (5µg) showed the highest resistance against the gram negative uropathogens, whereas Meropenem (10µg) and Cefixime (5µg) give highest sensitivity pattern against the gram positive uropathogenic isolates. This study demonstrated the significant data to monitor and compare with other science reports, the trend of antibiotic susceptibility of uropathogens and give us a specific guide line for empirical treatment of UTI patients.

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Conflict of interest

Authors declare that there are no conflicts of interests.

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