

# PREVALENCE OF BACTERIURIA, AND CHARACTERIZATION OF THEIR ANTIMICROBIAL SENSITIVITY PATTERN AND EFFLUX PUMPS ACTIVITY IN DIABETIC AND NON-DIABETIC UTI PATIENTS



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Apurbo Roy<sup>1</sup>, Rahena Yasmin<sup>2</sup>, Zimam Mahmud<sup>1</sup>, Farhana Tasnim Chowdhury<sup>1</sup> and Md. Mohasin<sup>1\*</sup>

<sup>1</sup>Infection and Immunity laboratory, Department of Biochemistry and Molecular Biology, University of Dhaka, Bangladesh

<sup>2</sup>Armed Forces Institute of Pathology, Dhaka, Bangladesh

## ABSTRACT

**Rationale:** Urinary tract infections (UTI) occur more frequently in patients with type 2 diabetes. In developing countries, impairments in the defense system, metabolic disorder during diabetes, and incomplete bladder emptying all contribute to the pathogenesis of UTI in diabetic patients. This comparative study is undertaken to reveal the prevalence of uropathogens and their antimicrobial resistance patterns, and to assess hematological biomarker in diabetic and non-diabetic UTI patients. **Methods:** The prevalence of uropathogens were tested in 40 diabetic and 40 non-diabetic UTI patients. The antimicrobial susceptibility pattern of bacterial isolates were assessed by the Kirby-Bauer disc-diffusion method. Moreover, the bacterial load, Gram-staining, biochemical and serological assays were performed using appropriate methods. **Results:** The findings showed that diabetic patients are more likely to UTI compared to non-diabetic patients. In terms of uropathogenic prevalence, 61% *Escherichia coli*, 34% *Klebsiella pneumoniae*, 2% *Staphylococcus aureus*, 2% *Pseudomonas aureginosa* and 1% others. The antimicrobial resistance profile demonstrated that most of the bacterial isolates were resistant to amoxicillin, ampicillin, erythromycin and methicillin but susceptible to azithromycin, chloramphenicol and ciprofloxacin. Moreover, the drug efflux pumps activity and bacterial burden were significantly high among antimicrobial resistant bacterial isolates. Besides, the C-reactive protein, Erythrocyte sedimentation rate, glycosylated hemoglobin A1C, neutrophils and lymphocytes were significantly high in diabetic UTI patients. **Conclusion:** This study found *E. coli* was the most frequent bacteria isolates, and this observation is consistent others. Diabetic UTI patients are more vulnerable with high bacterial burden and systemic inflammation.

**KEYWORDS:** Uropathogen, diabetic mellitus, antimicrobial resistance, efflux pumps

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CORRESPONDING AUTHOR: Dr. Md. Mohasin

Infection and Immunity laboratory, Department of Biochemistry and Molecular Biology, University of Dhaka, Bangladesh

Email: mohasin@du.ac.bd

## Introduction

Diabetic patients particularly type 2 diabetes mellitus (T2DM) are at increased risk of community-acquired and hospital-associated urinary tract infections (1-3). Hyperglycemic milieu in urinary tract may promote the growth of pathogenic bacteria (4). Besides, people with type 2 DM are immune-compromised, for example their immune system produces less amount of cytokines and shows dysfunctional phagocytosis by macrophages (5), which may facilitate to grow bacteria, hence people with diabetes are more vulnerable to infectious diseases as such urinary tract infections (UTIs) (1, 6, 7). However, previous studies showed no association between HbA1C level and risk of UTI among diabetic patients (7, 8). But other study shows UTIs alter glucose metabolism that impacts on blood sugar level (9), suggesting why T2DM patients are more prone to infectious diseases like UTIs. *E. coli*, *Klebsiella*, and *Proteus* are the other primary species that cause the majority of UTIs, along with *Enterobacter*, *Enterococcus*, and *Enterobacter* (10). Uncomplicated UTIs

do not cause death but sepsis, which results from UTI complications mostly responsible to death (11). Additionally, UTIs are more prone to have broad ranges of antimicrobial resistant uropathogens (12, 13). Severe sepsis or urosepsis have a mortality incidence of 20% to 40% (14). The current study is undertaken to assess the prevalence of bacteriuria, their antimicrobial resistance pattern as well as to evaluate the serological markers in diabetic and non-diabetic UTI patients.

## Method and Materials

### Sample Collection

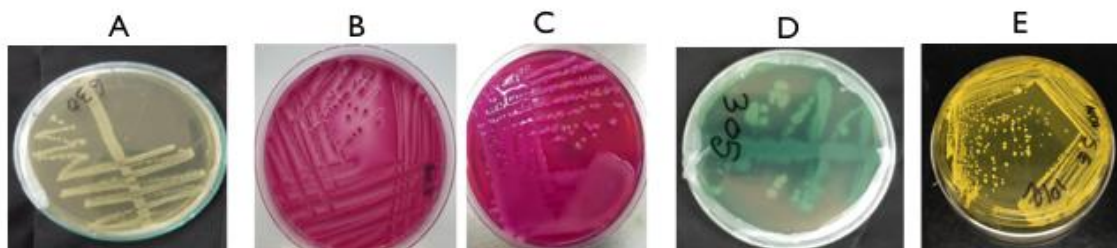
Clean-catch mid-stream 5 mL (circa) urine and 5 mL whole blood were collected from 40 diabetic and 40 non-diabetic UTI patients. All samples were processed within an hour after collection at the microbiology laboratory in Popular Medical Hospital, Dhaka. Subsequently, urine sample's bacterial isolates were grown in differential culture media and their antimicrobial resistance patterns were evaluated in the

department of Biochemistry and Molecular Biology, University of Dhaka.

#### Culture and identification of bacterial species

Three primary culture media (e.g. nutrient, differential and selective media) were used for the identification of bacterial isolates from urine specimens. For example, mannitol salt agar was used to isolate *S. aureus*, whereas the inability to ferment

mannitol confirms *S. epidermidis*. MacConkey agar has bile salts, and a basic dye (e.g. crystal violet), which hinder the growth of gram-positive organisms. This medium was used to observe and differentiate lactose fermenting bacterium (e.g. *E. coli*) and non-lactose fermenting bacterium (e.g. *K. pneumoniae*). Besides, Cetrimide agar was used to isolate and identify *P. aeruginosa* from clinical and non-clinical specimens.



**Figure 1.** A. Uropathogens on nutrient agar culture plate, B. MacConkey agar plate shows *E. coli* colonies, C. MacConkey agar plate shows *K. pneumoniae* colonies, D. Cetrimide agar plate shows the blue green colonies of *P. aeruginosa* and E. Mannitol agar media shows the yellow colonies of *S. aureus*.

**Table 1.** Uropathogens culture, differentiation and selection in diabetic and non-diabetic UTI patients

Name of the media	Type of Bacteria	Colony Characteristics
Nutrient media	All types of bacteria	
MacConkey agar media	<i>Escherichia coli</i> .	Red, round, small, solid
	<i>Klebsiella pneumoniae</i>	Pink, mucoid, large, semi-solid, sticky, moist.
Cetrimide agar media	<i>Pseudomonas aeruginosa</i>	Pigmented, green/blue-green
Mannitol agar media	<i>Staphylococcus aureus</i>	Yellow colonies with yellow zone

**Table 2.** Microscopic and biochemical characterization of bacterial isolates

Test	Procedure and observation	Findings
Gram Staining	Uropathogens (e.g. <i>S. aureus</i> , <i>K. pneumoniae</i> ) were stained on microscope slides using crystal violet and iodine. Subsequently counter staining with safranin. Gram positive bacteria retained deep violet to blue color, whereas gram-negative bacterium (e.g. <i>E. coli</i> ) lost violet and showed pink to red staining (15).	<i>S. aureus</i> is a Gram-positive bacterium
Catalase test	A few colonies of <i>S. aureus</i> were inoculated on a hydrogen peroxide solution (4% H <sub>2</sub> O <sub>2</sub> ) like a droplet on a microscopic slide.	<i>S. aureus</i> is catalase positive

**Antimicrobial susceptibility test**

Antimicrobial susceptibility was assessed by the modified disc diffusion (e.g. Kirby–Bauer) method. Urine specimens were culture in differential and selective media. The bacterial inoculum was prepared using liquid media (e.g. nutrient broth) by peaking up 3–5 cfu from pure culture. Subsequently, 100µl (e.g. equivalent to 0.5 MacFarland standard) of bacterial suspensions were inoculated on Mueller-Hinton agar plates through spread plate technique. Commercially available standard antibiotic discs were impregnated on Mueller-Hinton agar plates (Oxoid, UK). The standard discs were 10µg of Amoxicillin, 10µg of Ampicillin, 15 µg of Azithromycin, 30µg of Cephadrine, 30µg of Chloramphenicol, 5µg of Ciprofloxacin, 2µg of Clindamycin, 30µg of Doxycycline, 15µg of Erythromycin, (30µg), (30µg), 5µg of Methicillin and 30µg of Vancomycin. After 20 hours of incubation at 37°C, the zone of inhibition was measured and interpreted following the Clinical Laboratory Standard Institute guidelines (CLSI, 2017) as susceptible (S), intermediate (I), or resistant (R).

**Determination of efflux pump activity of bacterial isolates from urine specimens**

Both standard and bacterial isolates from urine specimens were separately cultured in appropriate culture media. Subsequently bacterial suspension (e.g. 10<sup>7</sup> cfu) was inoculated in 2µg/mL ethidium bromide (EtBr in PBS) and 0.25% glucose (in PBS) for a period of 30 minutes. Ethidium bromide containing buffer was replaced with EtBr-free PBS with or without glucose. The efflux pump activity was measured by

fluorescence plate reader at 530 nm excitation and 600 nm emission spectrums.

**Assessment of serological biomarkers**

Whole blood count, differential white blood cell count, HbA1C, ESR, CRC, random blood glucose levels were measured by automated cell counter, biochemical analyzer and appropriate serological assays in the popular diagnostic laboratory, Dhaka.

**Statistical Analysis**

All statistical analyses were performed by GraphPad Prism version 9.0. The Analysis of Variance (ANOVA) and Mann-Whitney test were performed. Values are expressed as mean ± SD (Standard deviation). Values of p<0.05 was considered as significant.

**Results****Baseline data of study participants**

A total of 40 diabetic and 40 non-diabetic UTI patients were participated in this study. The diabetic and non-diabetic UTI patients did not differ in terms of age and sex (p>0.05). The maximum study participants were female (60, 74%) for both diabetic patients and non-diabetic controls. The mean age (mean ±SD) was 50±9.0 for diabetic patients and 40±8.0 years for non-diabetic UTI patients. The serum glucose, HbA1c, neutrophils, lymphocytes and inflammatory markers (e.g. CRP and ESR) were significantly high among diabetic UTI patients compared to non-diabetic UTI patients (Table 3).

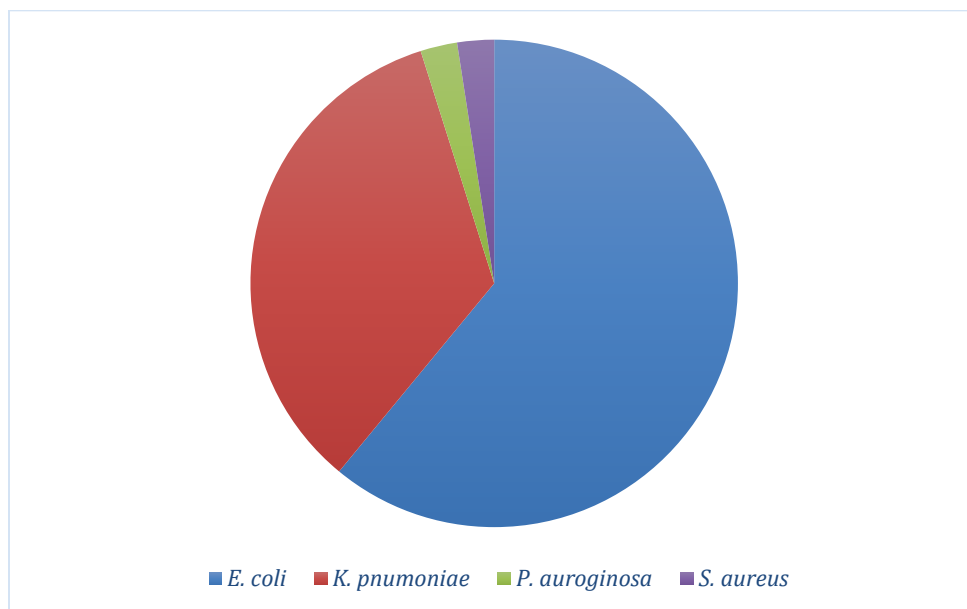
**Table 3.** Baseline data between diabetic and non-diabetic urinary tract infection patients

<b>Biomarkers</b>	<b>Diabetic patients with UTI</b>	<b>Non-diabetic UTI patients</b>	<b>p-value</b>
Serum glucose (random) (mMol/L)	10.2 ± 2.8	4.5 ± 2.1	0.001
HbA1C (%)	6.5 ± 0.7	5.0 ± 0.6	0.001
Total White blood cells (cumm)	8,200 ± 1025	7,650 ± 2050	0.001
Neutrophiles (%)	75.8 ± 6.0	61.2 ± 5.4	0.001
Lymphocytes (%)	45 ± 8.0	30.6 ± 4.2	0.001
ESR (mm/hour)	38.0 ± 6.0	37.0 ± 5.0	0.040
CRP (mg/L)	77 ± 10.3	53 ± 12.5	0.001
Hemoglobin (g/dL)	12.8 ± 0.8	12.5 ± 1.2	0.205

**Prevalence of uropathogens**

In terms of uropathogenic prevalence, Gram-negative *E. coli* was determined as the main class of bacteria (61%) associated with UTIs in the study cohort irrespective of diabetic or non-

diabetic UTI onsets. Among other bacterial isolates, *K. pneumoniae* accounted for about 34% of UTIs, followed by *P. aeruginosa* (2%) and *S. aureus* (2%) (Figure 2).



**Figure 2.** Pie chart shows the percentage of gram-positive and gram-negative bacterial isolates in urine specimens of UTI patients. In the gram-negative bacterial isolates, *E. coli*, *K. pneumoniae*, *P. aeruginosa* were detected, whereas, in the gram-positive isolates *S. aureus* and *S. epidermidis* were detected by differential culture method.

#### **Bacterial burden among diabetic and non-diabetic UTI patients**

The anatomical structure of female urethra and the immune-compromised diabetic comorbidity make them more prone to urinary tract infection (UTI). Among the bacterial isolates, the

bacterial total counts were significantly higher in diabetic UTI patients compared to non-diabetic UTI patients. The absolute bacterial burden were  $10^4$ - $10^5$  cfu/mL in diabetic UTI patients, whereas it was  $10^3$ - $10^4$  cfu/mL in non-diabetic UTI patients (Table 4).

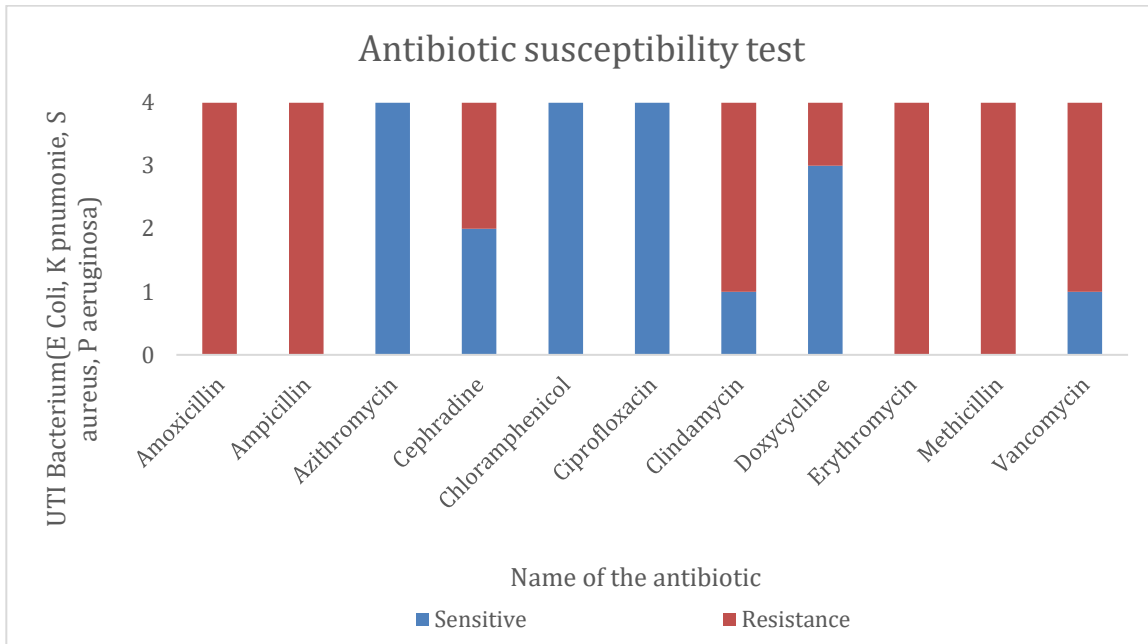
**Table 4.** Bacterial burden in urine specimens of diabetic and non-diabetic UTI patients

Bacterial species	Diabetic UTI patients (cfu/mL)	Non-diabetic UTI patients (cfu/mL)	<i>p</i> -value
<i>E. coli</i>	$1.2$ - $2.1 \times 10^5$	$1.0$ - $2.5 \times 10^4$	0.001
<i>K. pneumoniae</i>	$3.0$ - $3.5 \times 10^4$	$2.0$ - $4.0 \times 10^3$	0.001
<i>P. aeruginosa</i>	$2.1$ - $2.7 \times 10^5$	$2.2$ - $3.0 \times 10^4$	0.100
<i>S. aureus</i>	$3.5$ - $4.5 \times 10^4$	$3.0$ - $3.5 \times 10^4$	0.010
<i>S. epidermidis</i>	Non-detectable	$3.0 \times 10^3$	NA

#### **Antimicrobial Susceptibility patterns**

Antimicrobial susceptibility testing was performed against 11 clinically used antibiotics to determine their resistance profile. *E. coli* and *K. pneumoniae* showed maximum resistance to amoxicillin, ampicillin, erythromycin and methicillin.

Moreover, *P. aeruginosa* and *S. aureus* were observed resistance to  $\beta$ -lactam antibiotics, clindamycin and vancomycin. However, almost all of them showed sensitive to azithromycin, ciprofloxacin and chloramphenicol (Figure 3).

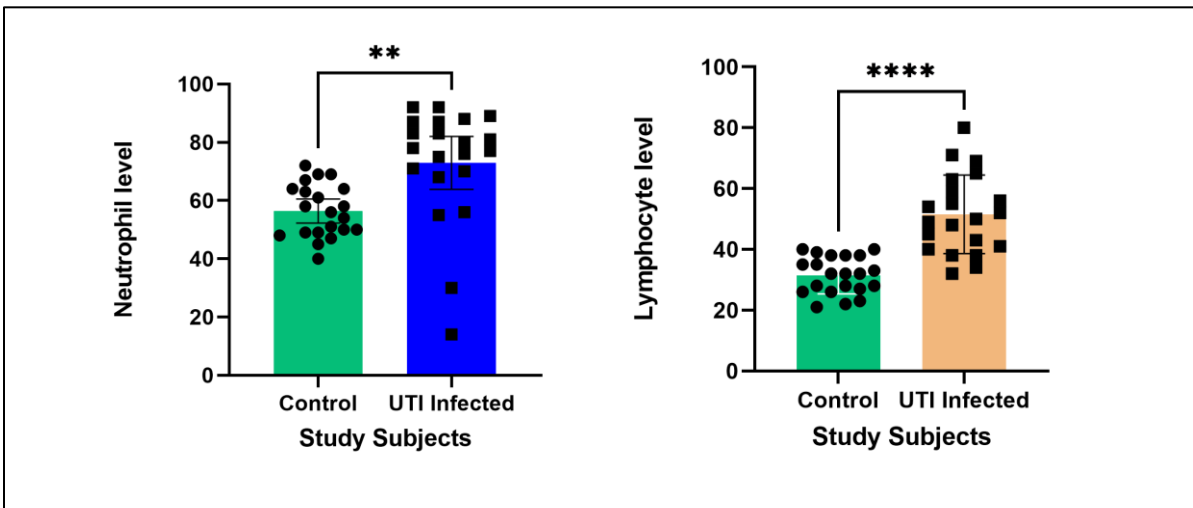


**Figure 3.** Antimicrobial resistance patterns in diabetic and non-diabetic UTI patients

**Levels of neutrophils and lymphocytes in UTI patients**

Apart from blood inflammatory markers, this study also evaluated total white blood cell and differential cell counts in UTI patients and healthy subjects. Results demonstrated that

neutrophil counts were significantly high in UTI patients compared to healthy volunteers (Figure 4A). Similarly, the absolute count of lymphocytes was also significantly high in UTI patients compared to healthy subjects (Figure 4B).

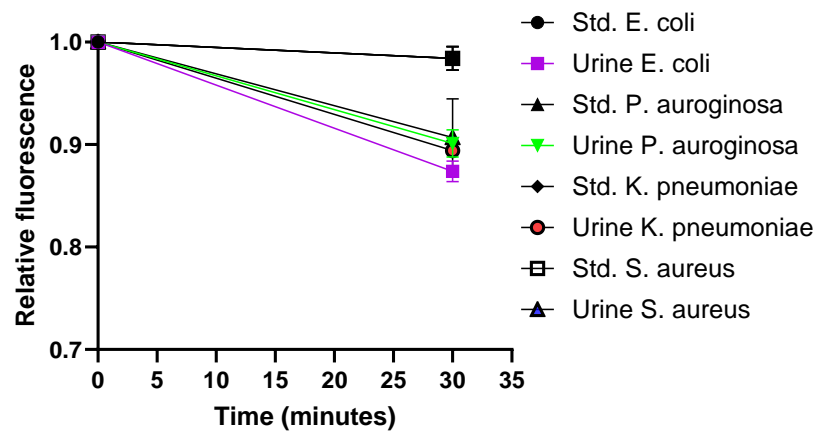


**Figure 4.** Percentage of neutrophils and lymphocytes in healthy and UTI volunteers

**Efflux pumps activity**

Previous study demonstrated that, efflux pump’s function has been associated with multidrug resistance phenotype in bacterial isolates (16). Among the antimicrobial resistance bacterial isolates, the function of efflux pumps was assessed by ethidium bromide efflux assay as described (17). Results demonstrated that the transport of the efflux pumps substrate

ethidium bromide was significantly high among antimicrobial resistance bacterial isolates compared to antimicrobial susceptible isolates (Figure 5). Moreover, the antimicrobial resistance patterns and the efflux pumps activity were positively correlated in UTI bacterial isolates (data not shown).



**Figure 5. Efflux pumps activity of uropathogens.** Figure shows the relative fluorescence of ethidium bromide (EtBr) in standard bacterial strains and bacterial isolates of UTI patients. The efflux activity of EtBr was measured at 0- and 30-minutes intervals by the loss of fluorescence of ethidium bromide from respective bacterial isolates. Std. stands for corresponding standard bacterial isolate.

## Discussion

Urinary tract infection (UTI) is a common cause of morbidity and mortality worldwide (18). Annually, around 150 million people are diagnosed with UTI globally, among them 35% are hospital-acquired infection (19). Previous studies demonstrated that *E. coli* is the most prevalent bacteriuria in pediatric UTI patients followed by *K. pneumoniae*, *Staphylococcus spp.*, *Proteus spp.*, *P. aeruginosa*, *Enterobacter spp.*, *Serratia spp.*, *Citrobacter spp.*, *Enterococcus spp.*, and *Streptococci agalactiae* (20, 21). The contributing factors for UTI in diabetic onsets are the functional impairment of immune cells (e.g. neutrophils and lymphocytes) (22, 23), metabolic disorder (4) and incomplete bladder emptying (24). In addition, previous study also demonstrated that diabetic UTI patients are more prone to infect with  $\beta$ -lactams, fluoroquinolone, carbapenem and vancomycin-resistant bacterial species (12, 13, 25, 26). The current study also found multidrug resistant bacterial isolates in UTI patients irrespective of diabetic or non-diabetic onsets. Furthermore, the bacterial burden, inflammatory mediators like ESR and CRP were significantly high in diabetic UTI patients. Besides the high levels of HbA1c, glucosuria and impairments of immune cells (22, 23) may facilitate to increase bacterial burden in diabetic UTI. In addition, multiple other factors are also involved to overwhelm antimicrobial resistance uropathogens in different clinical settings. Apart from incomplete-dose of antibiotic, alteration of antimicrobial target sites and acquisition of antimicrobial resistance genes, recently we have shown efflux pumps play a critical role to emerge amoxicillin resistant *S. aureus* (27). Moreover, other studies demonstrated that efflux pumps play essential roles in the development of multidrug resistance, biofilm formation, quorum sensing, and pathogenicity of bacteria, reviewed in (28). These observations were corroborated by our current findings, where we demonstrated the positive correlation between antimicrobial resistance and drug efflux activity among bacterial isolates in diabetic or non-diabetic UTI patients. Another study also demonstrated the positive correlation between antimicrobial profile and the efflux pump phenotype in UTI bacterial isolates (29). On the other hand, neutrophil-

lymphocyte ratio has a greater significance in predicting systemic infections (30). Previous mouse-model study demonstrated that UTI is associated with an increase recruitment of neutrophils to urinary tract (31). Moreover, this study also showed a defective neutrophil response to *E. coli* infection increases bacterial burden in mouse urinary tract (31). Besides neutrophilia is a prognostic marker of other infection or inflammation, such as pneumonia or a viral infection (32). In addition, some components of the immune system are also compromised by diabetic onsets (22, 23). These studies uphold the current and previous findings 'diabetic patients are more vulnerable to any kind of infections' like UTIs.

## Conclusion

Overall, the UTI patients were exposed with multidrug resistant bacterial isolates, which showed positive correlation with efflux activity. Moreover, the diabetic-associated UTI comorbidity might be responsible to increase urinary bacterial burden as well as systemic inflammation.

## Authors Contribution

AR have conducted and analyzed experiments. RY carried out efflux assay. ZM and FTC reviewed the manuscript. MM designed experiments and reviewed analysis with AR. MM wrote the manuscript with input of all authors.

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