Drug-resistant, biofilm-producing uropathogens isolated and characterized from urinary tract infected patients in Chattogram, Bangladesh

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ABSTRACT: Urinary tract infections are a major public health concern. The formation of biofilm is the prime cause of antibiotic resistance by uropathogens. This study was conducted for the determination and biochemical characterization of uropathogens with drug resistance and biofilm formation capacity among Urinary Tract infected patients in Chattogram, Bangladesh. The samples (n=109) had been collected from the patients of Chattogram Medical College and Meghna diagnostic center, Chattogram, Bangladesh. Pathogens were biochemically identified from 109 urine samples, and their antibiotic susceptibility was determined using a disk diffusion test. Biofilm formation capacity was observed and confirmed using the Congo red agar (CRA) method and sequence analysis of the *esp* and *cup A* genes. Among 109 urine samples, 50 (45.87%) patients were tested positive in culture with gram-negative bacteria, where the most predominant genus was *Escherichia coli* (54%), *Klebsiella spp.* (30%) and *Pseudomonas spp.* (16%), which also showed sensitivity against the antibiotic Imipenem and Netilmicin. Besides, almost 82% of isolates showed resistance to Ampicillin. An epidemiological study showed that females (60%) are more susceptible to UTI compared to children and males; where it also showed that the people (68%) of greater Chattogram in summer are mostly infected with uropathogens along with diabetes (32%). In the biofilm formation test, it was found that 36% of uropathogens were exopolysaccharide producers. But no isolate was detected in PCR analysis. The present study showed that Imipenem can be used as a suitable antibiotic to treat UTI.

KEYWORDS: urinary tract infection, antibiotic sensitivity, biofilm, epidemiological study

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Introduction

Urinary tract infection is an inflammatory reaction of the urinary tract epithelium caused by a wide range of pathogens` invasion, including Gram-negative and Gram-positive bacteria and fungi, usually accompanied by bacteriuria and pyuria (Gu *et al.*, 2020). It is found that UTI is one of the most prevalent diseases with diverse etiological agents, annually affecting 250 million, causing the death of 150 million people worldwide (Mama *et al.*, 2018; Azami *et al.*, 2019) and costing the global economy in excess of 6 billion US dollars (Akram *et al.*, 2007). For many decades, urine and the urinary bladder were considered to be sterile, but this theory has been changed as it was tested that there are many microorganisms living with us in our bladders (Thomas *et al.*, 2016).

The presence or absence of the symptoms marks the UTI as a symptomatic UTI or asymptomatic UTI. The urine will have a notable count of bacteria in these two common conditions. A count of $\geq 10^5$ to 10^6 is considered to confirm the presence of symptomatic or asymptomatic bacteriuria (ABU) during pregnancy (Harding *et al.*, 2002). ABU occurs in up to 6% of healthy individuals and 20% of elderly individuals. Many patients with ABU do not need treatment as in many cases the colonizing organism actually helps to prevent infection by other more virulent bacteria (Smaill and Vazquez, 2007). Postmenopausal women go through this infection depending on their age, health status, residential status (institutionalized or not), the presence of diabetes mellitus, history of catheterization, spinal cord dysfunction, and a history of antibiotic use. A single bacterial species is responsible for the noncatherized UTI where it can be possible to identify more than one species from the urine sample in case of structural abnormalities and catheterization. The increased use of catheters and instrumentation in these patients predisposes them to UTIs caused by Gram-negative rods such as *Proteus*, *Pseudomonas*, *Klebsiella*, and *Serratia*. In patients with diabetes mellitus, infections caused by *Klebsiella*, *Enterobacter*, and *Candida* are more common (Medina and Pino, 2019). Asymptomatic bacteriuria, symptomatic UTI, or UTI-related septicemia are all examples of symptomatic urinary system diseases. Bacteriuria $\geq 10^5$ CFU/ml is the most common preliminary count for laboratory diagnosis of UTI (Modi and Juthani, 2014). With the exception of a spike in young women aged 14-24 years old, the prevalence of UTIs

increases with age (Schmiemann *et al*, 2016). The prevalence in women over 65 years of age is about 20%, compared with approximately 11% in the overall population (Chu and Lowder, 2016). Between 50% and 60% of adult women have faced at least one UTI in their life, and close to 10% of postmenopausal women suggest that they had a UTI in the previous year (Alos, 2005). A biofilm can be defined as microorganisms bound to a surface of each other with the existence of an extracellular matrix made of secreted elements of the organisms and/or of components of the microorganisms themselves and it causes frequent and resistant device-related infections (Ramadan *et al*, 2021). Biofilms provide an environment for poor antibiotic penetration and horizontal transfer of virulence genes which favors the progression of Multidrug-resistant organisms (MDRO) (Katongole, 2020). Most uropathogens can survive in the form of biofilms which are caused for the recurrence and persistence of UTI. In this way, biofilms help microbial communities to propagate themselves in adverse conditions with minute nutrient availability and a strong immune system. Uropathogens show high antimicrobial resistance which is not only intrinsic resistance but also biofilm formation is found as a mechanism of resistance (Jamal, 2018). Approximately 80% of all infections with the urinary tract are for the involvement of biofilm-forming bacteria which can become a serious threat. Besides biofilm plays a major role in causing catheter-associated UTIs and recurrent UTIs (Agarwal and Radera, 2019). Various types of antibiotics have been used to treat UTIs. Antibiotic therapy is an effective and easy method to reduce the duration of symptoms. In the last decades, the expansive use of antibiotics has resulted in the emergence of antibiotic-resistant microorganisms and led to the event of antibiotic resistance (Bazzaz *et al* 2021).

The goals of our study are biochemical identification of UTI causal organisms, detection of multidrug resistance and biofilm formation capacities of those uropathogens, as well as an epidemiological study of the patients (to show the relationship in the context of seasonal, regional, age, and gender) and finally the relationship of UTI with other diseases.

Materials and Methods

Sample collection

The study population was drawn from patients from Chattogram Medical College and Meghna Diagnostic Center, Chattogram. The sample was collected from 109 patients having urine infections. At the same time, a conversation was done with the patients to fill up the questionnaire (**Supplementary file 1**) that contains information for the epidemiology study. The sample was collected seasonally as the time duration which covered two seasons (winter and summer).

Sample processing

A calibrated sterile micro wire loop for the semi-quantitative method was used for the plating and it has a 4.0 mm diameter designed to deliver 0.01 ml. A loopful of the well-mixed urine sample was inoculated into EMB agar. All plates were then incubated at 37ºC aerobically for 24 hours. The plates were then examined for bacterial growth. The bacterial colonies were counted and multiplied by 100 to give an estimate of the number of bacteria present per milliliter in the urine sample. The bacterial colony was determined as if any colony was significant equal to or in excess of 10,000 CFU/ml.

Phenotypic and biochemical characterization of bacterial isolates

The pure culture of bacterial isolates was identified based on cultural characteristics, gram staining, motility, and various biochemical tests as described in the Cowan and Steel's Manual for the identification of Medical Bacteria (Lower *et al.*, 2001; Barrow and Feltham, 2003). Isolates were biochemically analyzed for the activities of catalase, methyl-red test, citrate utilization, Voges-Proskauer (VP) test, and indole production according to the standard methods (Barrow and Feltham, 2003).

Determination of antimicrobial sensitivity pattern

The susceptibility of bacterial isolates to different antibacterial agents was determined *in vitro* by employing a modified disk diffusion test described by Kirby-Bauer (Bauer *et al.*, 1966). The procedure involved measuring the diameter of the zone of inhibition that results from the diffusion of the antibiotic agent into the medium surrounding the disc. Commercially available antimicrobial discs (OXOID Limited, Basingstoke, and Hampshire, England) were used for the test. The antimicrobial agent's Gentamicin (CN), Amoxycillin (AML), Levofloxacin (LEV), Ciprofloxacin (CIP), Imipenem (IMP), Nitrofurantoin (F), Netilmicin (NET), and Azithromycin (AZM) were tested for 50 isolates obtained from 109 urine samples of UTI patients.

Biofilm detection

The biofilm formation capacity was determined by the Congo red agar method and by PCR amplification of the *esp* gene for *E. faecalis* and *cup A* gene for *P. aeruginosa* (Eman and Abeer, 2015). Genomic DNA was extracted from the pure culture of isolated bacteria according to the alkaline lysis method (Sambrook and Russel, 2001). To identify the *esp* gene, the forward primer 5'-TTTTGGGGCAACTGGAATAG-3' and reverse primer 5'-TTCTGCCCCAGCAAATAGTC-3' were used in a primary PCR reaction to amplify about 515 bp of target DNA fragments. In addition, PCR reactions were performed by using forward primer 5'-AATTCGATGATCGCCTGTT-3' and reverse primer 5'-GCGATAGAGGTTGGTGTCGT-3' in order to amplify about 345bp target DNA fragments to identify *cup A* gene. All the handling of reaction mixtures and cycling conditions were the same as that of previously published standard protocols (Eman and Abeer, 2015). A PCR thermal cycler (NyxTechnik) was used in both primary and secondary PCR reaction cycles, and the products were analyzed by 1.5% agarose gel electrophoresis.

Epidemiological study

Epidemiological study of the patients has been done by filling up a questionnaire form which includes the formations (regional, age, sex, seasonal, and other related diseases) after a conversation with each patient of 109.

Results

Isolation of Bacteria

A total of 109 urine samples from UTI patients were examined during this study. It was found that out of 109 urine samples 50 patients tested positive for UTI (45.87%). Uropathogens were isolated and obtained in pure form according to their different morphological characteristics (colony size, shape, color, etc.) **(Table 1 and Figure 1).**

Table 1. Morphological Characteristics of Bacterial Isolates

The ID of Bacterial Isolates	Morphological Characteristics (color)
A3, A9, C6, N3	Pink
S1, S2, S3, S5, S5, S9, S10	Pink
P1, P7, P9, P10	Pink
A10, N1, N5, N6, N7	Colorless
P3, P4, S7	Colorless
A1, A2, A4, A5, A6, A7	Green metallic sheen
B2, B3, B4, B5, B6, B7, B10, C3, C4, C5, C7, C8, C9, D1, D2,	Green metallic sheen
N2, N4, P5, P6, P11, S8	Green metallic sheen

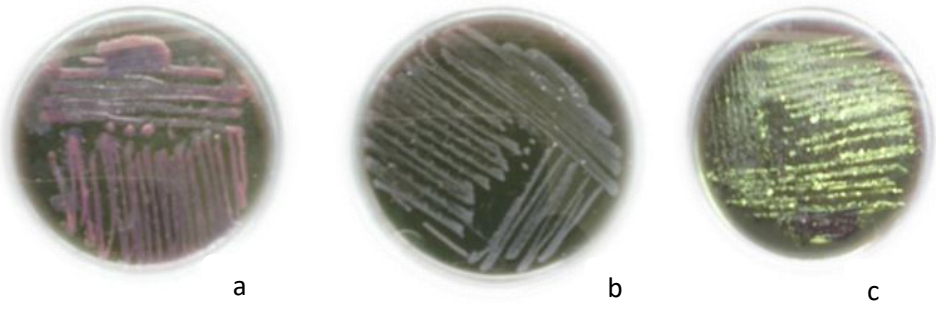

Figure 1. Color of the isolates on EMB media (a. pink colored isolate; b. colorless isolate; c. green metallic sheen colored isolate.

Biochemical Characterization of Uropathogens

After primary isolation of uropathogens in pure form according to their different morphological characteristics (colony size, shape, color, etc.) the secondary characterization of the isolates was performed on the basis of their biochemical characteristics. On the basis of phenotypic appearance in different media three kinds of uropathogens *Escherichia coli*, *Klebsiella spp.*, and *Pseudomonas spp.* were finally identified from 50 positive samples **(Table 2).**

Table 2. Biochemical test results

No. of Samples (out of 50 samples)	Biochemical Tests							Presumptive name of the isolates
	Gram staining	Catalase	Citrate Utilization	MR	VP	Indole	EMB	
28	Pink	Bubble formed	Blue	Red	-	Pink	Green metallic sheen	***Escherichia coli***
16	Pink	Bubble formed	Blue	-	Red	-	pink	***Klebsiella spp.***
6	Pink	Bubble formed	Blue	-	-	-	colorless	***Pseudomonas spp.***

Note: "–'' indicates no color change.

Drug Sensitivity Test of the Isolates

A drug sensitivity test was done with commonly used antibiotics. In this test, most of the isolates were sensitive to Imipenem and Netilmicin and showed resistance to other groups of antibiotics **(Table 3)**.

Table 3. Drug sensitivity test result

Name of Antibiotics	Sensitive Isolates	Resistance Isolates	% of sensitivity
Amoxycillin	9	41	18%
Levoflixacin	37	13	74%
Azithromicin	27	23	54%
Nitrofurantoin	37	13	74%
Gentamicin	**44**	**6**	***88%**
Ciproploxacin	27	23	54%
Netilmicin	**47**	**3**	***94%**
Imipenem	**50**	**0**	****100%**

**Shows the best results

Biofilm Detection

In this test, the biofilm formation capacity of the uropathogens was determined on the basis of color on the CRA media. Though all the isolates were non-biofilm formers, there are some isolates that produce pink color on the media, which means that they are exopolysaccharide producers **(Table 4).** For accurate determination of biofilm formation capacity, PCR analysis of PCR amplification of the *esp* gene for *E. faecalis* and the *cup A gene for P. aeruginosa* was done. But no isolate showed a positive result.

Table 4. Biofilm formation test by Congo Red Agar Method

No. of Isolates	Color	Result
A9, C6, D2, N1, N5,	Pink with darkening at the center	Weak slime producer
N6, N7, P1, P3, P7,	Pink with darkening at the center	Weak slime producer
P9, S1, S2, S3, S5,	Pink with darkening at the center	Weak slime producer
S7, S9, S10	Pink with darkening at the center	Weak slime producer
A1, A2, A3, A4, A5,	White	Non-biofilm former
A6, A7, A10, B2, B3	White	Non-biofilm former
B4, B5, B6, B7, B10	White	Non-biofilm former
C3, C4, C5, C7, C8,	White	Non-biofilm former
C9, D1, N2, N3, N4,	White	Non-biofilm former
P4, P5, P6, P10, P11,	White	Non-biofilm former
S8, S6	White	Non-biofilm former

Epidemiological Study Related to UTI

During the research work, an epidemiological study was performed where data is presented here as the prevalence of UTI on the basis of Age, Gender, Region, Disease, and Season, respectively. The distribution of UTIs between children and adults was 42% and 58% respectively **(Figure 2a).** The prevalence of UTI is more frequent and high in females (60%) than males (40%) **(Figure 2b**). In the Chattogram division prevalence of UTI is high in the Chattogram district **(Figure 2c).** The epidemiological study also showed that UTI-infected people are suffering from various diseases like Diabetes, High Blood Pressure (HBP), Low Blood Pressure (LBP), and Kidney Diseases (KD), where the prevalence rate is highest in diabetic patients (32%) **(Figure 2d).** On the basis of a seasonal view, it can be said that UTIs are more frequent in summer (56.81%) than in winter (38.46%) **(Figure 2e).**

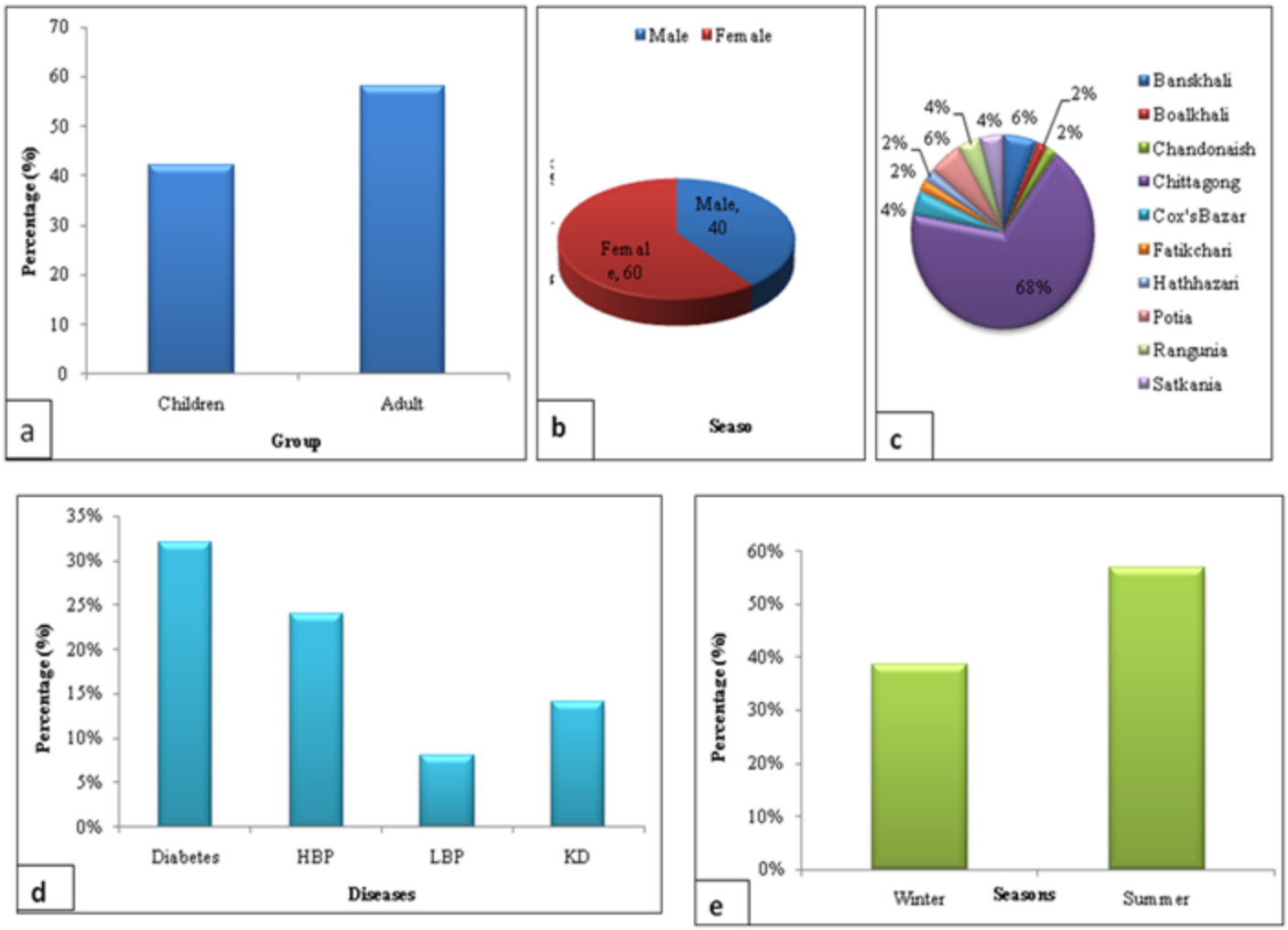

Figure 2 (a-e). a. Distribution of UTI on the basis of age; b. Distribution of UTI on the basis of Gender; c. Distribution of UTI in a different region of Chittagong; d. Distribution of UTI with other diseases; e. Prevalence of UTI in two Seasons.

Discussion

Bangladesh is a densely populated country with poor sanitary knowledge among people. As a result, infectious diseases are a common phenomenon in this country (Rehman *et al*, 2014). In the present study, uropathogens were found in males and females including the poor, middle, and upper classes. We studied 109 urine samples and found UTI in about 45.87% of cases where a higher incidence of UTI was noticed in women (60%) and adults (58%) compare to men (40%) and children (48%), respectively (Figure 2a and 3b). The high frequency in women may be related to the anatomical structure of a genitourinary system. A short urethra, proximity of the urethra to the anus, and vaginal colonization by fecal flora members may facilitate ascending infection into the bladder (Singh and Bijoyalakshmi, 2016). Lack of toilet training knowledge may be a reason for UTI susceptibility in the children.

Chattogram is one of the largest districts in Bangladesh with favorable geological conditions that may help pathogens to grow and survive. The people of Chattogram city are more vulnerable to UTI (68%) than other Upazillas **(Figure 2c).** This occurrence may be due to the unhealthy and unhygienic lifestyle of the city people. The poor's lack of sanitary knowledge may lead to UTI.

Several research findings have shown the correlations between diabetes and UTI in the form of data proof. Long-term effects on the genitourinary system caused by diabetes mellitus. Our findings say that about 32% of people are diabetes patients along with UTI (**Figure 2d**). Our study also shows that other diseases - High Blood Pressure (24%), Low Blood Pressure (8%), and Kidney Diseases (14%) are also found in UTI patients. Kidney diseases may be also an effect of UTI as they are considered part of the urinary system. Bacterial infection may also cause kidney diseases as uropathogens can move in the system. Findings say that people are mostly affected with UTI in summer (56.81%) than in winter (38.46%) (**Figure 2e**). Ambient temperature may be a risk factor for UTI. Also, changes in urination during the summer may increase the risk of UTI as more sweating causes less urination and thus results in the multiplication of microorganisms among people (Rustom *et al*, 2020).

Uropathogens were identified by the standard loop method. They were primarily grown on selective media EMB. On the basis of phenotypic appearance in media three kinds of uropathogens *E. coli*, *Klebsiella spp.*, and *Pseudomonas spp.* were identified from 50 positive samples (**Table 1 and Figure 1**). These positive samples further go for confirmation test by standard biochemical methods (**Table 2**).

Due to the abuse of antibiotics, microbial agents are being resistant. Microorganisms' drug resistance ability is considered a growing health problem in the treatment of infectious diseases (Prah *et al*, 2019). Again the lifestyle of people may be another reason for being affected by UTI. The drug sensitivity pattern of the uropathogens was done according to the disk diffusion method. This experiment was applied to each of the positive samples to observe their drug sensitiveness. It was found that 22 samples (44%) showed multi-drug resistant as they show their resistance to three or more three groups of drugs in this experiment. Imipenem and Netilmicin showed 100% and 94% sensitiveness respectively against all of the 50 samples (**Table 3**).

In our study, it was found that people had taken different antibiotics before their urine infection. About 40% of the patients said that they had taken antibiotics for urine infection. This finding may indicate that frequently taken antibiotics may be one of the reasons for multi-drug resistant (MDR) uropathogens. Antimicrobial resistance is also associated with high mortality rates and high medical costs and has a significant impact on the effectiveness of antimicrobial agents. MDR provokes obstruction in disease control by intensifying the possibility of spread of resistant pathogens, thus, declining the efficacy of treatment and, hence, resulting in a prolonged time of infection in patients. The cost of treatment is also increased due to MDR as the pathogens have become resistant to commercially available drugs, which has triggered the use of more expensive therapies. So, without consulting a doctor, the consumption of any antibiotic may cause health hazards. Patients should take antibiotics after proper diagnosis of the pathogens. As the present investigation showed most of the predominant uropathogens were sensitive to Imipenem and Netilmicin so it can be said that these antibiotics will be a better treatment than other antimicrobial drugs.

Biofilms can be defined as a group of microbial colonies that are attached to a biotic or abiotic surface by producing an extra-polymeric matrix of their own or from the host's components. Several changes occur during the biofilm formation process, in which genetic, physiological, and metabolic differences are observed compared to their planktonic (Hola *et al*, 2021). The CRA test for biofilm detection is easier and faster to perform than other phenotypic tests. A color scale was adopted to compare the results of the CRA test. In the CRA test colonies presents a bright black and dry black color which was classified as positive and those presenting a red, pink, or Bordeauxcolor as weak slime producers (Kırmusaoğlu, 2019). In the current study, 18 isolates (36%) showed such a pink color in the biofilm test (Table 4), while others showed white. It means that though these isolates are non-biofilm former, they may have the capacity to produce exopolysaccharides that act as a matrix for biofilm formation. Furthermore, it can also be said that the drug resistance capacity of the uropathogens may not be for the biofilm formation. They may utilize other mechanisms to escape from the antibiotic.

The process of bacterial biofilm formation has erupted in recent years through several experiments and studies of the molecular genetics of biofilm formation have started to find the driving forces behind the transition to the biofilm mode of existence (Jefferson, 2004). As studies show that both *esp* gene and *cupA* gene are responsible for the production of the extracellular polymeric substances for biofilm progression (Taglialegna *et al*, 2020; Vallet *et al*, 2004). To understand the molecular basis of biofilm, our study further went for the detection of the *esp* gene of *E. faecalis* and the *cup* gene of *pseudomonas.* The 18 isolates of weak slime producers, were selected for molecular diagnosis using PCR. They include one *E. coli*, ten *Klebsiella*, and seven *Pseudomonas*. However, no positive results were found in this study. So it can be highlighted that although these isolates are genetically not capable to produce and propagate biofilm although they slightly produce polysaccharides for the attachment on the surfaces temporarily.

Conclusion

Among the people of greater Chattogram, females are at higher risk of UTI, especially compare to males. It has been also found that diabetic patients in the summer seasons are also at high risk for this infection. Imipenem and Netilmicin were found in this study to be the most effective antibiotics against UTI. Though biofilm formation capacity was not detected it plays an important role in the drug resistance capacity of UTI. However, necessary steps and regular monitoring should be taken to create consciousness among people to prevent earlier urinary tract infection disease in Bangladesh. A large-scale epidemiological investigation is necessary to get further information about the prevalence, drug resistance, and biofilm formation capacity of uropathogens in Bangladesh.

Authors Contribution

This work is a product of the intellectual effort of the whole team and all members have contributed in diverse magnitude to the analytical methods used, the research concept, the experiment design, and the manuscript preparation.

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Supplementary Figure

Supplementary data associated with this article can be found in the online version at: doi.org/10.3329/brc.v8i2.60641

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