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Determination of Antibiotic resistance pattern of Biofilm producing Pathogenic Bacteria associated with UTI

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Abstract:
The present study was aimed to ascertain the antimicrobial resistance of pathogenic bacteria associated with urinary tract infections (UTIs). *Escherichia coli* was found to be the most prevalent uropathogen (75.0%), followed by *Klebsiella pneumoniae* (12.5%), *Pseudomonas* spp. (8.33%) and *Staphylococcus aureus* (4.16%). Isolated bacteria (n=24) were characterized for their sensitivity to commonly prescribed antibiotics. Although Congo red binding assay indicated that all of the isolates (100%) were able to produce exopolysaccharide, only 70.83% of the isolated bacteria produced biofilm in tube adherence assays. Biofilm producing bacteria showed higher level of resistance against all of the antibiotics tested except to amikacin (30µg), meropenem (10µg) and piperacillin-tazobactam (100/10µg). Meropenem was found to be the most effective (87.5%) antibiotic and pathogens were mainly resistant to ciprofloxacin (62.5%). Multidrug resistance was observed for 91.6% of the isolates against ≥ 1 antibiotic of which 50% of the isolates showed resistance against ≥ 5 antibiotics.

Keywords: uropathogen, biofilm, antibiotic resistance

Introduction

Urinary tract is frequently subjected to colonization by various normal flora some of which may act as opportunistic pathogens [1]. Urinary Tract Infection (UTI) is a broad term used to describe a group of disease resulting from the microbial colonization of the urinary tract [2]. It includes a variety of infections such as urethritis, cystitis and acute and chronic pyelonephritis [2]. UTIs continue to pose a serious threat, with millions of cases being reported yearly showing high recurrence rates and possibility of developing into chronic diseases [3]. UTIs remain common in Bangladesh and other developing countries, affecting individuals of different age groups [4] It can also have a significant impact on the socioeconomic lives of affected individuals, contributing largely to the increase in the consumption of antimicrobial drugs [4, 5].

Infection may originate from the bladder and can progress to the kidneys resulting in renal failure [3]. Complications may arise in those who have an indwelling bladder catheter, which increases the possibility of bacteriuria [6]. The causes of UTI include poor perineal hygiene, pregnancy, urinary tract obstruction, urethral reflex, catheterization, sexual intercourse, contraception use, history of UTIs and diabetes [1, 7]. The anatomical structure of the female genitourinary tract makes them more susceptible to the disease, particularly during pregnancy [4, 7, 8]. UTIs are one of the more common types of nosocomial infections

accounting for 25 to 40% of the infection [9].

Observations of patients suffering from catheter associated urinary tract infection have revealed increased rates of biofilm formation among microbes that attach and grow on the surface of catheters [9]. Biofilms appear on any surface as an aggregation of bacteria enclosed in a polysaccharide matrix. It often leads to diverse bacterial subpopulations resulting from differential gene expression and aids bacteria in developing resistance to both host defense mechanisms and antibiotics [11]. Biofilms may have significant consequences in both medical and non-medical settings such as, food and water processing and distribution systems [9].

This study was aimed to determine the antibiotic sensitivity patterns of pathogenic bacteria isolated from urine cultures in both planktonic state and in biofilms.

MATERIALS AND METHODS

Isolation of pathogenic bacteria

Pathogenic bacteria were isolated from mid-stream clean catch urine samples, collected from suspected UTI patients. Urine samples were inoculated on Blood agar and MacConkey agar (Himedia Laboratories Ltd., India) using a measured loop to determine the colony forming units (cfu/ml urine). Samples that showed $>10^3$ cfu/ml of either one or two types of bacteria were subsequently identified for antibiotic susceptibility assay. Isolates were further identified using colony morphology and biochemical tests [11]. A total of 24 bacterial isolates were included in this study.

Preparation of inoculum

Freshly cultured isolates were used for the antibiotic sensitivity assay. Individual bacteria were propagated in tryptic soy broth (TSB) from a fresh culture plate at 37°C and adjusted to 0.5 McFarland standard before inoculating on Mueller Hinton agar (MHA, Oxoid, UK).

Antibiogram

Bacterial susceptibility to antimicrobial agents was determined *in vitro* by using agar disc diffusion method following CLSI guidelines [12]. Commercially available antibiotic discs that were used from Oxoid, UK included amikacin (AMK) 30 µg, ciprofloxacin (CIP) 5 µg, clindamycin (CM) 2 µg, rifampicin (RA) 5 µg, cefuroxime (CFX) 30 µg, trimethoprim/sulfamethoxazole (TS) 1.25/23.75 µg, erythromycin (ERY) 15 µg, Gentamicin (GM) 10 µg, penicillin 10 units, amoxicillin 500 µg, amoxicillin/clavulanate (AMC) 20/10 µg, cefixime (CMF) 30 µg, cefepime (CPM) 30 µg, vancomycin (VAN) 30 µg, linezolid (LNZ) 30 µg, ceftriaxone (CRO) 30 µg, ceftazidime (CAZ) 30 µg, meropenem (MRP) 10 µg, piperacillin-tazobactam (PTZ) 100/10 µg, nitrofurantoin (NIT) 300 µg, carbenicillin (CB) 100 µg, tobramycin (TOB) 10 µg and aztreonam (ATM) 30 µg.

Determination of minimum inhibitory concentration

Minimum inhibitory concentrations (MIC) of ciprofloxacin, ceftriaxone and tobramycin were determined using broth dilution method [12]. Antimicrobial agents were dissolved into appropriate solvents to prepare stock solutions from where they were diluted to a range of concentrations viz. 64 µg/ml, 32 µg/ml, 16 µg/ml, 8 µg/ml, 4 µg/ml, 2 µg/ml, 1 µg/ml, 0.5 µg/ml, 0.25 µg/ml, 0.125 µg/ml, and 0.065 µg/ml by 2 fold serial dilution in Mueller Hinton Broth (MHB).

Determination of biofilm formation by tube adherence method

Brain heart infusion broth (BHIB, Oxoid, UK) was inoculated with the desired bacteria in 2 ml volume to have a final concentration of 10^6 cfu/ml

and incubated at 37°C for 48 hours. The supernatant was discarded and the glass tube was stained by 0.1% safranin solution. Tubes were washed three times with phosphate buffered saline (PBS) and dried. Tubes showing thin line of films on the tube walls were considered positive. Tubes showing only a single line of stained ring at the liquid-air interface were considered as a negative result [14].

Congo red method

Congo red agar was prepared by supplementing brain heart infusion broth (BHIB, 37 gms/L) with agar no.1 (10 gms/L) and congo red stain (0.8 gms/L). Congo red was prepared as a concentrated aqueous solution and autoclaved separately at 121°C for 15 minutes from other medium constituents. Agar media was cooled at 55°C and congo red stain was added to achieve a final concentration of 0.8 gms/L. Plates were inoculated and incubated aerobically for 24 to 48 hours at 37°C. Black colonies with a dry crystalline consistency were considered as positive. Pink colonies and colonies with occasional darkening at the centers of colonies were considered as weak slime producers [15]. All experiments were performed in triplicate.

Minimum regrowth concentration (MRC)

Bacterial cells were inoculated at a concentration of 10^6 cfu/ml in tubes containing 2 ml of TSB and incubated at 37°C for 24 hours for biofilm formation. Planktonic cells were decanted and the biofilms were washed with sterile PBS. Tryptic soy agar (TSA, Himedia Laboratories, India) containing different concentrations of antibiotics between 64 µg/ml and 0.065 µg/ml in two fold serial dilutions were added to the tubes containing biofilms. Tubes were incubated at 37°C for 24 hours for regrowth of the bacteria. The minimum concentration for which visible growth was inhibited was considered as MRC for that bacterium against the particular antibiotic tested [13].

Quality control

Escherichia coli ATCC 25922 and *Staphylococcus aureus* ATCC 25923 were used as control strains in this study.

Results

The proportion of isolated UTI pathogens is shown in Table 1. Amongst the 24 isolates *Escherichia coli* was found to be the most frequently isolated organism (75.0%) followed by *Klebsiella pneumoniae* (12.5%), *Pseudomonas* spp. (8.33%) and *Staphylococcus aureus* (4.16%).

Table 1: Percentage distribution of uropathogens in UTI patients

Uropathogenic organisms	Number (%)
Escherichia coli	18 (75.0 %)
Klebsiella pneumoniae	3 (12.5 %)
Pseudomonas spp.	2 (8.33 %)
Staphylococcus aureus	1 (4.16 %)
Total	**24 (100 %)**

In this study we determined biofilm production capability of all of the isolates both by tube adherence method (TAM) and exopolysaccharide production by congo red binding assay (CRA). It was found that all isolates showed exopolysaccharide production in CRA (Table 2). However, a relatively lower proportion of *Escherichia coli* (76.47%), *Klebsiella pneumoniae* (33.33%) and *Pseudomonas* spp. (50%) showed positive result in TAM.

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Table 2: Determination of biofilm formation using tube adherence (TAM) method and congo red agar method (CRA)

Bacterial organisms	Biofilm producers (%)		Total
	TAM	CRA	
Escherichia coli	76.47 %	100 %	17
Klebsiella pneumoniae	33.33 %	100 %	3
Pseudomonas spp.	50 %	100 %	2
Staphylococcus aureus	100 %	100 %	1
Streptococcus agalactiae	100 %	100 %	1
Total	**70.83 %**	**100 %**	**24**

The summary of the antibiotic sensitivity patterns of the isolated bacteria which produced biofilm is shown in Table 3.

Table 3: Percentage of distribution of antibiotic resistance pattern of biofilm and non-biofilm producing bacteria

Antibiotics	Resistance pattern, n (%)	
	Biofilm	Non-biofilm
Amikacin, 30 µg	0 (0.0%)	1 (4.1%)
Ciprofloxacin, 5 µg	11 (45.8%)	3 (12.5%)
Cefuroxime, 10 µg	8 (33.3%)	4 (16.6%)
Trimethoprim/sulfamethoxazole, 1.25/23.75 µg	5 (20.8%)	3 (12.5%)
Gentamicin 10 µg	5 (20.8%)	2 (8.3%)
Amoxicillin/Clavulanic acid, 20/10 µg	7 (29.1%)	4 (16.6%)
Cefixime, 30 µg	7 (29.1%)	4 (16.6%)
Cefepime, 30 µg	7 (29.1%)	5 (20.8%)
Ceftriaxone, 30 µg	7 (29.1%)	4 (16.6%)
Meropenem, 10 µg	0 (0.0%)	1 (4.1%)
Piperacillin-tazobactam, 100/10 µg	0 (0.0%)	1 (4.1%)
Nitrofurantoin, 300 µg	2 (8.3%)	2 (8.3%)

All isolates were used to determine the MIC and MRC against selected antibiotics *viz*. ceftrioxone, ciprofloxacin and tobramycin following broth dilution method (Table 4). MIC was determined against planktonic cells whereas MRC was determined by challenging the bacteria with different concentrations of antibiotics after formation of biofilm. All the bacteria tested showed higher levels of resistance after the formation of biofilms than those of planktonic cells.

Table 4: Determination of MIC and MRC values of uropathogens against ceftriaxone, ciprofloxacin and tobramycin

Sample no.	Ciprofloxacin (µg/ml)		Ceftrioxone (µg/ml)		Tobramycin (µg/ml)	
	MIC	MRC	MIC	MRC	MIC	MRC
Escherichia coli	>64	>64	2	>64	64	>64
Pseudomonas spp.	>64	>64	8	>64	8	>64
Escherichia coli	2	>64	4	>64	4	>64
Escherichia coli	2	>64	2	>64	1	>64
Escherichia coli	2	>64	2	>64	2	>64
Staphylococcus aureus	>64	>64	2	>64	>64	>64
Streptococcus agalactiae	>64	>64	>64	>64	>64	>64
Escherichia coli	2	>64	2	>64	2	>64
Pseudomonas spp.	0.25	>64	2	>64	1	>64
Escherichia coli	1	>64	2	>64	2	>64
Escherichia coli	2	>64	2	>64	0.25	>64
Klebsiella pneumoniae	2	>64	2	>64	1	>64
Klebsiella pneumoniae	0.5	>64	16	>64	2	>64
Escherichia coli	>64	>64	32	>64	2	>64
Escherichia coli	2	>64	2	>64	2	>64
Escherichia coli	2	>64	2	>64	2	>64
Escherichia coli	2	>64	2	>64	2	>64
Escherichia coli	2	>64	2	>64	2	>64
Escherichia coli	2	>64	2	>64	2	>64
Klebsiella pneumoniae	2	>64	2	>64	0.5	>64
Escherichia coli	2	>64	2	>64	2	>64
Escherichia coli	0.25	>64	2	>64	1	>64
Escherichia coli	2	>64	32	>64	2	>64
Escherichia coli	2	>64	2	>64	2	>64

Multidrug resistant bacteria are defined as bacteria resistant to more than one antibiotic. The incidence of multi-drug resistance (MDR) among UTI isolates was also determined in this study (Table 5). Both gram negative and gram positive bacteria showed multi drug resistance. It was found that the incidence of multidrug resistance was higher in ciprofloxacin resistant groups. According to this study, 22 (91.6%) isolates showed resistance against ≥ 1 antibiotic and 2 (8.3%) isolates showed resistance against ≥ 8 antibiotics.

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Table 5: Multiple drug resistantce patterns of isolated bacteria

Number of drugs	Number of isolates showing resistance
≥ 1	22 (91.6%)
≥ 2	15 (62.5%)
≥ 3	13(54.1%)
≥ 4	13 (54.1%)
≥ 5	12 (50.0%)
≥ 6	10 (41.6%)
≥ 7	5 (20.8%)
≥ 8	2 (8.3%)

Discussion

Amongst all infectious diseases, urinary tract infections (UTIs) represent one of the most common diseases in both developed and developing countries causing a large number of morbidity in different age groups [16]. It has been noticed that the etiological characteristics of UTI and their antibiotic resistance patterns may vary in different geographic locations [17]. Therefore it is essential to study local etiological agents and their antibiotic susceptibility patterns for appropriate treatment and eradication of UTIs. Biofilm production by pathogenic bacteria of the urinary tract may further complicate treatment options by showing high level of resistance to antibiotics [18].

This study includes 24 pathogenic strains of which *Escherichia coli* (75%) was the major pathogen followed by *Klebsiella* spp. (12.5%) and *Pseudomonas* spp. (8.3%). The finding of this study is in concordance with that of others showing *E. coli* (74%) as the predominant organism followed by *Klebsiella* spp. (17.7%) and *Pseudomonas* spp. (2.5%) [19]. In another study it was found that *E. coli* was found to be the major uropathogen (80%) in community acquired infection followed by *Staphylococcus saprophyticus* (10-15%), *Klebsiella*, *Enterobacter* and *Proteus* spp. [20].

It usually takes 48 hours to conduct culture and sensitivity tests and deliver report on a urine sample, consequently the majority of the community-acquired UTI (CAUTI) treatment decision is empirically based on the assumption that commonly encountered pathogens are present and antibiotics are prescribed accordingly. Culture and sensitivity tests also cost more than the antibiotic treatment itself which further complicates empiric treatment of CAUTI even more. Most of the time treatment of UTI goes without prior knowledge of antibiotic susceptibility of individual pathogen. Therefore it is important to conduct continuous surveillance of antibiotic susceptibility of uropathogens.

In this study it was observed that the infected patients were mostly women (79.16%). A similar finding was reported by others [21, 22]. A range of antibiotics were applied on the isolated bacteria on both biofilm producing and non-biofilm producing bacteria in this study. The most effective antibiotics against isolated bacteria were found to be meropenem (95.9 %), amikacin (95.9 %) and piperacillin-tazobactam (95.9 %) followed by nitrofurantoin (83.4 %), gentamicin (70.9%). Isolates showed highest resistance against ciprofloxacin (58.3%) amongst the applied antibiotics. Similar results were found in studies conducted in Iran [23, 24]. Another study has reported a worldwide increase in antibiotic resistance over the last few decades [25]. A decline in the activity of ciprofloxacin would be especially problematic in view of the ability of gram negative bacteria to acquire resistance to all other classes of antimicrobials [26]. A significant increase in resistance of pathogenic strains to SXT has been found worldwide [27].

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It is well established that bacteria forming biofilms show distinct characteristics in terms of resistance to antibiotic treatment. Bacteria residing in the biofilms can persist for long period of time and can demonstrate dramatic increase in their resistance to antibiotics [10]. These bacteria can also be released in adjacent liquid medium and act as a source of infection in catheter patients [28].

In this study we found that multidrug resistance was higher in those bacteria that were able to produce biofilms. Previous studies found that 80% of the strains producing biofilm were multidrug resistant [29]. Antimicrobial activity of three antibiotics, ceftrioxone, ciprofloxacin and tobramycin were determined as MIC on planktonic cells and was found to be ≥2 µg/ml. However, MRC values for these same antibiotics were found to be ≥ 64 µg/ml. Once bacteria form biofilm, it limits diffusion of antibiotics and sometimes can adapt and form protected phenotypes [30, 31, 32]. It has been reported that both extended spectrum beta-lactamase (ESBL) and non-ESBL producing microorganisms demonstrated higher levels of antibiotic resistance when they form biofilm but the activity of the beta-lactam antibiotics increased when they were applied in combination with other chemicals such as ethylene diamine tetra acetic acid (EDTA) and sulbactam [33]. It is expected that antibiotic combinations may be useful for treatment of multidrug resistant microorganisms to enhance the effect of individual antibiotics.

Conclusion

Escherichia coli was most frequently isolated bacteria in this study of which 76.47 % were capable of producing biofilms. Biofilm producing bacteria are often found to be nosocomial and associated with the devices used for patients. Quinolones appeared to be the least active drug on the studied uropathogens. There was a strong correlation between formation of biofilms and antibiotic resistance pattern. However, this study was done on a limited number of samples which may be increased in future studies to conduct a broad scale study for the sake of preparing a guideline for patients having infection with pathogens capable of producing biofilms.

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