Antiobiotic resistance pattern of biofilm-forming uropathogens isolated from catheterised patients in Pondicherry, India

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RESEARCH

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Abstract

Background

Microbial biofilms pose a public health problem for persons requiring indwelling medical devices, as micro-organisms in biofilms are difficult to treat with antimicrobial agents. Thus the present study includes biofilm formation and antibiotic resistance pattern of uropathogens in hospitalised patients with catheter associated urinary tract infections (UTI).

Method

This prospective analysis included 100 urine samples from catheterised patients with symptoms of UTI over a period of six months. Following identification, all isolates were subjected to antibiotic sensitivity using modified Kirby-Bauer disc diffusion method. Detection of biofilms was done by tube adherence method and Congo red agar method.

Results

E.coli was found to be the most frequently isolated uropathogen 70%, followed by *Klebsiella pneumoniae* 16%, *Pseudomonas aeruginosa* 4%, *Acinetobacter spp* 2%, coagulase negative *Staphylococci* 6% and *Enterococci spp* 2%. In the current study 60% of strains were in vitro positive for biofilm production. Biofilm positive isolates showed 93.3%, 83.3%, 73.3% and 80% resistance to nalidixic acid, ampicillin, cephotaxime and cotrimoxazole, respectively, compared to 70%, 60%, 35%, 60% resistance showed by biofilm non-producers for the respective antibiotics. Approximately 80% of the biofilm producing strains showed multidrug resistant phenotype

Conclusion

To conclude E.coli was the most frequent isolate, of which 63% were biofilm producers. The antibiotic susceptibility pattern in the present study showed quinolones were the least active drug against uropathogens. The uropathogens showed the highest sensitivity to carbapenems. The next best alternatives were aminoglycosides. Significant correlation between biofilm production and multi-drug resistance was observed in our study.

Key Words

Biofilm, Uropathogens, Tube adherence method, Congo red agar method

Background

Biofilms are microbial communities of surface-attached cells embedded in a self-produced extracellular polymeric matrix.[1] They can cause significant problems in many areas, both in medical settings (e.g. persistent and recurrent infections, device-related infections) and in non-medical (industrial) settings (e.g. biofouling in drinking water distribution systems and food processing environments).

Biofilms have major medical significance as they decrease susceptibility to antimicrobial agents. The decreased susceptibility to microbial agents within a biofilm arises from multiple factors, including physical impairment of diffusion of antimicrobial agents, reduced bacterial growth rates, and local alterations of the microenvironment that may impair activity of the antimicrobial agent. Furthermore, the proximity of cells within a biofilm can facilitate plasmid

exchange and hence enhance the spread of antimicrobial resistance.[2]

Biofilm is the predominant mode of growth in aquatic ecosystems and, as such, plays a central role in the pathogenesis of Catheter Associated Urinary Tract Infections (CAUTI). Most aspects of the diagnosis, treatment, and prevention of CAUTI are influenced by the tenacity of biofilm-associated uropathogens. The biofilm mode of living is a highly advantageous response of the micro-organisms to the environmental stresses of the urinary tract environment.

Thus the present study was done to study the antibiotic resistance pattern of biofilm forming uropathogens in patients with catheter associated urinary tract infections.

Method

This prospective, analytic study was done for a period of six months. A total of 100 urine samples were collected from patients of all age groups and both the sexes with a urinary catheter for at least two days suffering from symptoms of UTIs. The study was approved by the Institutional Human Ethical Committee (IHEC) and informed written consent was taken from the patients before collection of samples. Samples were collected under complete aseptic conditions with a sterile syringe from the distal end of the urinary catheter and transferred to a sterile urine container and transported immediately to the laboratory without any delay.

Urine samples were inoculated on Blood Agar, MacConkey's agar and Cystine lactose electrolyte deficient (CLED) medium with a calibrated loop to determine colony-forming units (CFU). All specimens with bacteriuria of $>10^3$ colony-forming units (cfu)/mL urine (which defines CAUTI) of one or two organisms were analysed to determine their causative uropathogens and their antibiotic susceptibilities.[3]

Identification of isolates was done by colony morphology, gram staining and standard biochemical tests. Bacterial susceptibility to antimicrobial agents was determined by the Kirby Bauer disk diffusion method on Muller-Hinton agar. Isolates were categorised as susceptible, moderately susceptible, and resistant, based upon interpretive criteria developed by the Clinical and Laboratory Standards Institute (CLSI).[4] Antibiotic discs (Hi-Media) ampicillin (10µg), cefuroxime (30µg), cefotaxime (30µg), amikacin (30µg), gentamicin (10µg), cotrimoxazole (1.25/23.75µg), norfloxacin (10µg), netilmycin (30µg), nitrofurantoin (300µg), nalidixicacid (30µg), ciprofloxacin (5µg), erythromycin (15µg), oxacillin (1µg), linezolide (30µg), imipenem (10µg) and vancomycin (30 µg) were used for antimicrobial susceptibility tests.

Detection of biofilms was done by tube adherence method and Congo red agar method.

Tube adherence method by Christensen et al[5]

Suspension of tested strains was incubated in the glass tubes containing Brain Heart Infusion Broth (broth) aerobically at the temperature of 35°C for a period of two days. Then the supernatant discarded and the glass tube was stained by 0.1% safranin solution, washed with distilled water three times and dried. A positive result is defined as the presence of a layer of stained material adhered to the inner wall of the tubes. The exclusive observation of a stained ring at the liquid-air interface should be considered negative

Congo red agar method by Freeman et al[6]

Suspension of tested strains was inoculated onto a specially prepared solid medium --- brain heart infusion broth (BHI) supplemented with 5% sucrose and Congo red. The medium was composed of BHI (37 gms/L), sucrose (50 gms/L), agar no.1 (10 gms/L) and Congo red stain (0.8 gms/L). Congo red was prepared as concentrated aqueous solution and autoclaved at 121°C for 15 minutes, separately from other medium constituents and was then added when the agar had cooled to 55°C. Plates were inoculated and incubated aerobically for 24 to 48 hours at 37°C.

A positive result was indicated by black colonies with a dry crystalline consistency. Weak slime producers usually remained pink; though occasional darkening at the centres of colonies was observed. A darkening of the colonies with the absence of a dry crystalline colonial morphology indicated an indeterminate result. The experiment was performed in triplicate and repeated three times.

Biofilm positive by any one these two methods were taken as positive for biofilm production.

Quality control

The following international reference strains were used as controls: the biofilm producers *S. epidermidis* ATCC 35984 (positive control) and the non-biofilm producers *S. epidermidis* ATCC 12228 (negative control).

Statistical analysis

The statistical analysis was done by taking percentage and simple ratios.

Results

The number and percentage of each uropathogen isolated from catheterised urine samples are shown in Table 1.

Table 1: Percentage distribution of uropathogens from catheter urine samples

Bacterial organisms	Frequency (%) of Isolation
Escherichia coli	70
Klebsiella pneumoniae	16
Pseudomonas aeruginosa	4
Acinetobacterlwoffi	2
Coagulase negative Staphylococci	6
Enterococci	2
Total	**100**

Out of these 100 strains, *E.coli* was found to be the most frequently isolated pathogen 70%, followed by *Klebsiella spp* 16*%, Pseudomonas aeruginosa* 4%, *Acinetobacter spp* 2%, *coagulase negative* Staphylococci 6% *and Enterococci* 2%.

Table 2: Screening of 100 urinary isolates for biofilm formation

Bacterial organisms	Biofilm Producers (%)	Biofilm non-producers (%)	Total
Escherichia coli	44 (63%)	26 (37%)	70
Klebsiella pneumoniae	10 (63%)	6 (37%)	16
Pseudomonas aeruginosa	3 (75%)	1 (25%)	4
Acinetobacter lwoffi	1 (50%)	1 (50%)	2
Coagulase negative staphylococci	2 (33%)	4 (67%)	6
Enterococci spp	--	2 (100%)	2
Total	**60**	**40**	**100**

In the current study 60% of strains were *in vitro* positive for biofilm production and 40% were negative for biofilm production (Table 2).

A comparison of slime production by both methods showed that there was complete agreement between two methods in 84 of the total 100 isolates (Table 3).

Table 3: Results of methods for detecting biofilm production

No of Tests	Congo red method	Christensen method
44	+	+
40	--	--
10	+	--
6	--	+

A total of 54 (90%) isolates were biofilm positive by Congo red agar method and 50 (83.3%) isolates were biofilm producers by Christensen's method.

Table 4: Antibiotic resistance pattern of biofilm and non biofilm producers

Antibiotics	Resistance		
	Biofilm positive isolates (n=60)	Biofilm negative isolates (n=40)	Resistance of all isolates (n=100)
Ampicillin(10µg)	50 (83.3%)	24 (60%)	74 (74%)
Amikacin(30µg)	16 (26.6%)	4 (10%)	20 (20%)
Cephotaxime (30µg)	44 (73.33%)	14 (35%)	58 (58%)
Cotrimoxazole	48 (80%)	24 (60%)	72 (72%)
Norfloxacin (10 µg)	48 (80%)	24 (60%)	72 (72%)
Gentamicin (10 µg)	22 (36.66%)	18 (45%)	40 (40%)
Netilmycin (30 µg)	46 (76.66%)	22 (55%)	68 (68%)
Nalidixicacid (30 µg)	56 (93.33%)	24 (70%)	84 (84%)
Nitrofurantoin (300µg)	28 (46.66%)	12 (30%)	40 (40%)

The overall percentage of resistance observed among all the isolates including biofilm producers and biofilm non producers are shown in Table 4.

There was 80% resistance to nalidixic acid, ampicillin, cephotaxime and cotrimoxazole, respectively, compared to 70%, 60%, 35%, 60% resistance showed by biofilm non-producers for the respective antibiotics. Multidrug resistance pattern of the biofilm producing isolates is shown in Table 5.

Table 5: Multiple drug resistant pattern of biofilm producers

Multiple drug combinations	Number of isolates showing resistance	Per cent
A, Co, Na, Nx, Ctx	48	80
A, Na, Nx, Ctx	8	13.3
Co, Na, Nx, Ctx	4	6.7
A, Co, Na, Ctx	8	13.3

A=Ampicillin, Co=Cotrimoxazole, Ctx=Cephotaxime, Na=Nalidixic acid, Nx=Norfloxacin

There was a significant correlation between biofilm production and resistance to multiple antibiotics such as ampicillin, co-trimoxazole, cefotaxime, nalidixic acid and norfloxacin. Out of the total 60 strains isolated, 48 (80%) strains were multidrug resistance phenotype.

Discussion

CAUTI is the most common nosocomial infection in hospitals and nursing homes, comprising >40% of all institutionally acquired infections.[7-9] The relevance of biofilm to CAUTI is that a foreign body, such as an indwelling urethral catheter, connecting a normally sterile, hydrated body site to the outside world will inevitably become colonised with microorganisms.[10]

The present study showed out of these 100 strains, *E.coli* was the most frequently isolated pathogen 70%, followed by *Klebsiella spp* 16%, *Pseudomonas aeruginosa* 4%, *Acinetobacter spp* 2%, *coagulase negative* Staphylococci 6% *and Enterococci* 2% which was similar to the findings of Hassin[11] showing *E.coli* (74%) as the predominant organism followed by *Klebsiella spp* 17.7% & *Pseudomonas spp* 2.5%. Ronald in his study found that *E. coli* remains the predominant uropathogen (80%) in community acquired infections followed by *S. saprophyticus* (10-15%), *Klebsiella, Enterobacter, Proteus spp*. [12]

Escherichia coli is responsible for more than 80% of all UTIs and causes both symptomatic UTI and asymptomatic bacteriuria (ABU).[13, 14] The ability of uropathogenic *E. coli* (UPEC) to cause symptomatic UTI is associated with the expression of a variety of virulence factors, including adhesins (e.g., type 1 and P fimbriae) and toxins (e.g., hemolysin).[14,15]

Although there were few samples yielding more than one type of bacteria in culture, since it was insignificant in number, those isolates were excluded from our study. Biofilms may be composed of a single species or multiple species, depending on the device and its duration of use in the patient. Urinary catheter biofilms may initially be composed of single species, but longer exposures inevitably lead to multispecies biofilms.[16]

In the current study, 60% of strains were in vitro positive for biofilm production. A similar study showed 73% of biofilm production by uropathogens from UTI.[17] Significant production of biofilm was seen in 44 (63%) isolates of *E. coli* whereas Sharma *et al.* have reported a similar rate of biofilm production (67.5%) in *E.coli* and 70.3% biofilm production was more in patients with catheters.[18]

It is well accepted that "Sessile" bacteria within biofilms are physiologically quite distinct from unattached, "planktonic" bacteria. However the nature of the bacterial biofilm adherent to the catheter can be appreciated by aspiration cultures of the planktonic bacteria being released from the biofilm. Planktonic bacteria are continually shed from the colonised catheter into the residual urine that is always present around the tip and balloon of the catheter.[19]

We investigated antibiotic resistance patterns of biofilm producers and non-biofilm producers against drugs currently used in therapy of UTI. The investigated biofilm strains displayed relatively high resistance against tested antibiotics than non-biofilm producers. Resistance to four antibiotics such as ampicillin (83.3% vs. 60%), cephotaxime (73.3% vs. 35%), norfloxacin (80% vs. 60%) and nalidixic acid (93.3% vs. 70%) was comparatively higher among biofilm producers than non-biofilm producers. Bacterial biofilms are often associated with long-term persistence of organisms in various environments and they display dramatically increased resistance to antibiotics. [20]

The present study also showed significant correlation between biofilm production and multidrug resistance, where 80% of strains producing biofilm were multidrug resistant phenotypes.[21] Therapy against UTI should be guided by antimicrobial susceptibilities as increasing numbers of urinary isolates are developing resistance to commonly used antibiotics. Increasing antimicrobial

resistance of uropathogens has led to reconsideration of traditional treatment of recommendations in many areas.

Microbial biofilms have been associated with a variety of persistent infections which respond poorly to conventional antibiotic therapy. This also helps in the spread of antibiotic resistant traits in nosocomial pathogens by increasing mutation rates and by the exchange of genes which are responsible for antibiotic resistance. Antibiotic therapy against device associated biofilm organisms often fails without the removal of the infected implant. An elevated expression of the efflux pump is another mechanism for the development of antibiotic resistance in biofilm bacteria. Physiological heterogeneity is another important characteristic which is observed in biofilm bacteria. This phenomenon affects the rate of growth and metabolism of the bacteria and is reflected by interbacterial quorum signals, the accumulation of toxic products and the change in the local micro environment. These so-called persister cells are not resistant to antibiotics per se, but become resistant when associated with the biofilm.[22]

Conclusion

In conclusion, E.coli was the most frequent isolate, of which 63% were biofilm producers. The antibiotic susceptibility pattern in the present study showed quinolones were the least active drug and the uropathogens showed the highest sensitivity to carbapenems and aminoglycosides. Significant correlation between biofilm production and multidrug resistance was observed in our study. As biofilm production was detected in many of our isolates, it is necessary to establish standard guidelines on the care of catheter for all units in the hospital with a view to preventing nosocomial infections associated with the device in patients.

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CONFLICTS OF INTEREST

The authors declare that they have no competing interests.

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