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The efficacy of immediate versus delayed antibiotic administration on bacterial growth and biofilm production of selected strains of uropathogenic Escherichia coli and Pseudomonas aeruginosa

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ABSTRACT

Purpose: The treatment of urinary tract infections (UTI) with antibiotics is commonly used, but recurrence and antibiotic resistance have been growing and concerning clinicians. We studied whether the rapid onset of a protective biofilm may be responsible for the lack of effectiveness of antibiotics against selected bacteria.

Materials and Methods: Two established uropathogenic *Escherichia coli* strains, UTI89 and CFT073, and two *Pseudomonas aeruginosa* strains, PA01 and Boston-41501, were studied to establish a reliable biofilm formation process. Bacterial growth (BG) was determined by optical density at 600 nm (OD 600) using a spectrophotometer, while biofilm formation (BF) using crystal violet staining was measured at OD 550. Next, these bacterial strains were treated with clinically relevant antibiotics, ciprofloxacin HCl (200 ng/mL and 2 µg/mL), nitrofurantoin (20 µg/mL and 40 µg/mL) and ampicillin (50 µg/mL) at time points of 0 (T0) or after 6 hours of culture (T6). All measurements, including controls (bacteria -1% DMSO), were done in triplicates and repeated three times for consistency.

Results: The tested antibiotics effectively inhibited both BG and BF when administered at T0 for UPEC strains, but not when the antibiotic administration started 6 hours later. For *Pseudomonas* strains, only Ciprofloxacin was able to significantly inhibit bacterial growth at T0 but only at the higher concentration of 2 µg/mL for T6.

Conclusion: When established UPEC and *Pseudomonas* bacteria were allowed to culture for 6 hours before initialization of treatment, the therapeutic effect of selected antibiotics was greatly suppressed when compared to immediate treatment, probably as a result of the protective nature of the biofilm.

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INTRODUCTION

Uropathogenic *Escherichia coli* (UPEC) is the leading cause of urinary tract infection (UTI) and, in the United States alone, health care costs for UTI surpass $1.5 billion per year (1-4). UTIs rank among the most common bacterial infectious diseases encountered in clinical practice, with the occurrence of UTI in the United States estimated at 12-50% for women and 3% for men (3, 5). In patients with bladder or catheter-associated infections, the primary etiologic agents associated

with UTIs are strains of E.coli and *Pseudomonas aeruginosa* (P.aeruginosa) respectively. Therefore, this study focused attention on well-established strains of these two bacteria.

Currently, antibiotics represent the most effective treatment against UTI. However, some patients exhibit recurrent infections and/or appear to develop resistance to antibiotics (4, 6). Several studies have indicated that the production of bacterial biofilm, a large bacterial community that forms following bacterial adhesion and colonization to surfaces and in which bacteria are held together by exopolysaccharides (7) secreted by the bacteria, contributes to antibiotic resistance (8). A mature biofilm can contain a community of cells and provide a matrix for chemical signaling and message relay between individual cells (5, 9). Thus, we were interested in determining the role of biofilm production by E.coli and P.aeruginosa bacteria strains as one of the major contributors to antibiotic resistance. To study the biofilm formation by bacteria treated with antibiotics, two established strains of E.coli (UTI89 and CFT073) and two well-characterized strains of P.aeruginosa (Boston-41501 and PA01) were selected.

We first determined the time course of the growth of each bacteria strain and biofilm under different culture conditions. We also examined whether different pH conditions, consistent with the range of urine pH in humans, had any impact on bacterial growth and biofilm formation. Based on these optimized culture conditions, we examined the effect of clinically relevant antibiotics on the bacterial growth and biofilm formation. Furthermore, we evaluated whether different treatment schedules of antibiotics have any impact on bacterial growth and biofilm formation.

MATERIALS AND METHODS

Study Design

Following the evaluation of the impact of biofilm performance under different culture conditions and pH ranges, biofilm formation obtained from established strains of E.coli and P.aeruginosa was assessed in the presence of commonly used antibiotics in the treatment of urinary tract infections. The timing of antibiotic treatment on biofilm formation and bacterial growth was also studied to determine the effect of delayed therapy.

Bacterial strains

E.coli UTI89 and E.coli CFT073 were obtained from Harry Mobley (University of Michigan); both strains have been sequenced. E.coli UTI89 is a prototypic cystitis isolate (1) and E. coli CFT073 is a prototypical UPEC isolate cultured from blood and urine of a patient with pyelonephritis (10). P.aeruginosa-Boston 41501 was purchased from American Type Culture Collection (Manassas, VA), and P.aeruginosa-PA01 was obtained from Kevin McIver; only PA01 has been sequenced. Antibiotics (Ampicillin, Ciprofloxacin and Nitrofurantoin) were purchased from Sigma-Aldrich (St. Louis, MO) and used at the following concentration ranges: Ciprofloxacin HCl (Cipro: 200 ng/mL and 2 µg/mL), Nitrofurantoin (Nitro: 20 µg/mL and 40 µg/mL), and Ampicillin (Amp: 50 µg/mL). DMSO was used as a solvent to dilute all the antibiotics. The final concentration of DMSO was 0.1%. Biofilm formation was not affected at this low concentration of DMSO.

Determination of Minimum Inhibitory Concentration (MIC) by the microdilution method

MIC for E.coli and P.aeruginosa strains were assessed by varying concentrations of Ciprofloxacin, Nitrofurantoin, and Ampicillin assessing bacterial growth through CFU counts as previously described (11). An adjusted inoculum of the overnight growth organism was introduced into LB broth containing serial dilutions in 96 well plates, from 0.0015 µg/mL to 100,000 µg/mL of an initial antibiotic solution, with approximately $5x10^5$ CFU/mL inoculum. Results were observed after 18 hours of incubation at 37°C. The MIC was defined as the lowest concentration to inhibit visible growth (11).

Bacterial culture condition and growth assay

Each bacteria was cultured to log phase at 37°C in 3 mL Luria-Bertani (LB, pH 7.4) broth with no supplementation. Bacterial suspensions were diluted 1:100 and plated 100 µl into a 96-well plates containing LB broth with 1% glucose (Sigma-Aldrich). The plates were incubated at 37°C with agitated shaking (AS) at 250 rpm) or no agitation

(static environment (SE)) for 6-72 hours or incubated in LB broth with different pH environments (pH 5 to 8) for 24-48 hours. Bacterial growth was determined by optical density at 600 nm (OD_{600}) using a spectrophotometer. Furthermore, serial dilutions of culture were performed and plated for colony forming units (CFU).

For adjusting different pH in LB broth, either hydrochloric acid or sodium hydroxide was used to either decrease or increase the pH, respectively, to 5, 6, 7 and 8. Media was then filtered through a 0.2 µm sterile syringe filter (Corning, Inc., Corning, NY). Each bacterium was added into aliquots of sterile pH-adjusted media and pH was measured using pH indicator strips (EM Science, Cherry Hill, NJ) before and after incubation.

Crystal violet staining for biofilm assay

We followed the methodology for biofilm assay as previously described (8) with minor modifications. Briefly, after determining bacterial growth density, the supernatant was discarded, and attached bacteria and biofilm were washed twice with sterile water. The attached biofilm was stained with 100 µl of 0.4% crystal violet (Sigma-Aldrich) for 15 minutes at room temperature. The wells were rinsed twice with sterile water, then air dried. The biofilm-retained crystal violet was eluted with 100 µl of 100% ethanol and incubated for 5 minutes at room temperature on a shaker (175-200 rpm). The OD_{555} was determined using a spectrophotometer.

Statistical analyses

Each experiment was repeated three times for consistency, with samples in triplicate. Data were presented as the mean ± SEM, and the differences between two groups compared by the Student's t test. Statistical significance ($p<0.05$) was shown by asterisks (*) above the bars on the graphs.

RESULTS

Determination of culture condition for bacterial growth and biofilm production

As shown in Figure-1A, the time course study indicated that the growth of both E.coli strains, UTI89 and CFT073, reached a plateau in 6 hours. The AS condition was more favorable for UTI89 while no significant difference was noted for CFT073 in either condition. For CFT073, the biofilm production gradually increased under either condition, while UTI89 formed biofilm by 6 hours which stayed fairly consistent thereafter (Figure-1B). For P.aeruginosa Boston-41501, the bacterial growth reached a plateau around 24 hours with no significant difference observed in AS or SE conditions (Figure-1C). In contrast, P.aeruginosa PA01 continued to grow in AS condition from 24 to 72 hours, as compared to SE condition (Figure-1C).

Overall, both Boston-41501 and PA01 P.aeruginosa strains showed more robust biofilm production than both E.coli strains (Figures 1C and D). Noticeably, under AS condition, Boston-41501 exhibited tremendous biofilm production capability in a time-dependent manner even when bacterial growth reached a plateau at 24 hours (Figure-1D). For Boston-41501, although the biofilm production pattern remained similar in both AS or SE conditions, the biofilm production in the SE condition was significantly lower than in the AS condition (Figure-1D). For PA01 strain in AS conditions, it appeared that biofilm production was established at 6 hours and did not increase further over time (Figures 1C and D). In contrast, for PA01 strain under SE condition, bacterial growth reached a plateau within 24-48 hours but biofilm continued to increase in a time-dependent manner (Figures 1C and D). Taken together, with the exception of PA01, AS conditions appeared to be more favorable for both growth and biofilm production for all the bacterial strains tested in this study.

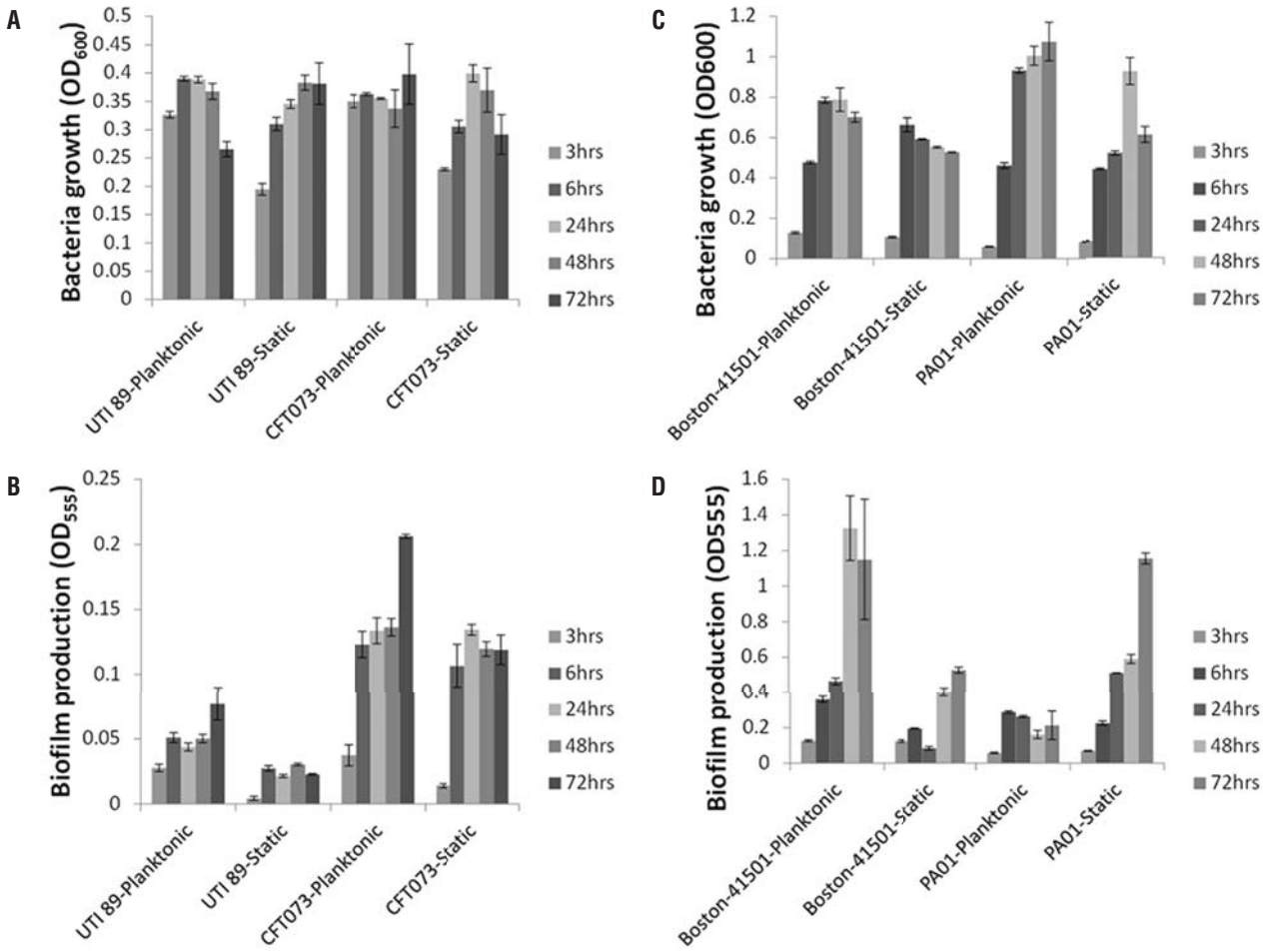
Effect of pH

As indicated in Figure-2, the different pH levels did not affect the growth or biofilm formation of our E.coli and P.aeruginosa strains, with the exception of the more acidic conditions (pH 5). P.aeruginosa strains were able to bring pH levels back to a consistent level (neutral pH) across the study at 48 hours.

Effect of antibiotics on bacterial growth and biofilm production

To assess the role of antibiotic concentration on biofilm, we initially determined the MIC of an-

Figure 1 - Time course analysis of growth and biofilm patterns.

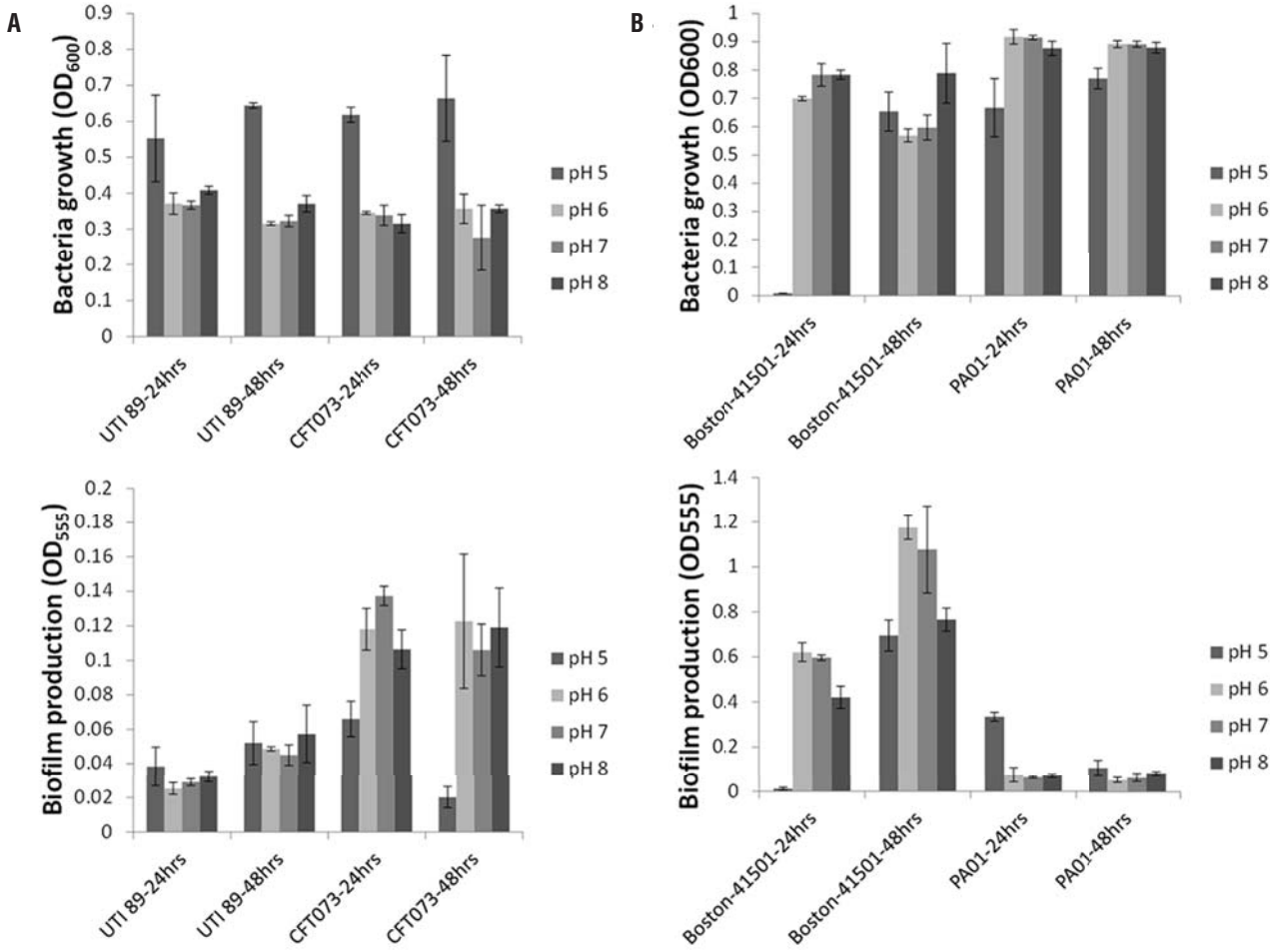


tibiotic tested for our selected strains of E.coli and P.aeruginosa. Antibiotics used in this study were chosen based on their common clinical use. For Ciprofloxacin, MIC was 100 µg/mL for all selected strains. For Ampicilin, MIC was 100,000 µg/mL for P.aeruginosa PA01, 10,000 µg/mL for P.aeruginosa Boston-41501, and 1,000 µg/mL for both E.coli strains. For Nitrofurantoin, MIC was 400 µg/mL for both P.aeruginosa (Boston-41501 and PA01) strains, 100 µg/mL and 20 µg/mL for UTI 89 and CFT 073 respectively. The dosage reported in urine is 2 µg/mL (8, 12) for Ciprofloxacin and between 25 and 400 µg/mL for Nitrofurantoin. Therefore, to make this study relevant, we used concentrations of antibiotics similar to the concentrations found in urine, which are significantly lower than the MIC. Ampicilin was used primarily as a negative control in a similar range used for the other two antibiotics.

As shown in Figures 3A and B, all antibiotics were able to significantly inhibit both bacterial growth and biofilm production for both UTI89 and CFT073 at 24 hours when antibiotics were added at Time 0. For UTI89 and CFT073, Ampicilin appeared to be less effective than Ciprofloxacin or Nitrofurantoin for inhibiting bacterial growth and biofilm production. Taken together, for UTI89 and CFT073, antibiotics can effectively inhibit both growth and biofilm production when administered at baseline (Table-1).

For P.aeruginosa strains, only Ciprofloxacin was able to significantly inhibit bacterial growth while Nitrofurantoin and Ampicilin failed to inhibit bacterial growth even when these antibio-

Figure 2 - Effect of different pH levels on bacterial growth and biofilm production over 48 hours.



tics were added at Time 0 (Figure-4A). Interestingly, for both strains, Ciprofloxacin at 200 ng/mL or 2 µg/mL and Ampicilin at 50 µg/mL significantly inhibited biofilm production. Surprisingly, Nitrofurantoin increased biofilm production in both strains (Figure--4B). Antibiotics also effectively inhibited both growth and biofilm production of P.aeruginosa when they were administered at baseline (Table-1).

Subsequently, E.coli and P.aeruginosa strains were cultured for 6 hours prior to receiving antibiotic treatment for an additional 24 hours. As shown in Figures 5A and B, E.coli bacterial growth and biofilm production were not inhibited by any of the antibiotics.

For Boston-41501, Ciprofloxacin still remained effective for both bacterial growth and biofilm production inhibition when it was added 6 hours after the initial plating of bacteria (Figures 6 A and B). However, a significant inhibition of biofilm production by Ciprofloxacin was only detected at 2 µg/mL for the PA01 strain. Nitrofurantoin was not only an inefficient antibiotic for either P.aeruginosa strain but its presence stimulated their biofilm production (Figure-6B). Ampicillin failed to inhibit bacterial growth of both P.aeruginosa strains (Figure--6A) but it showed significant inhibitory effect on biofilm production (Figure-6B).

DISCUSSION

Recurrent urinary tract infections represent a major clinical challenge as they affect a

Figure 3 - Effects of antibiotic treatment on E.coli from Time 0. Asterisks (*) show statistical significance as compared to bacteria with no treatment (P<0.05).

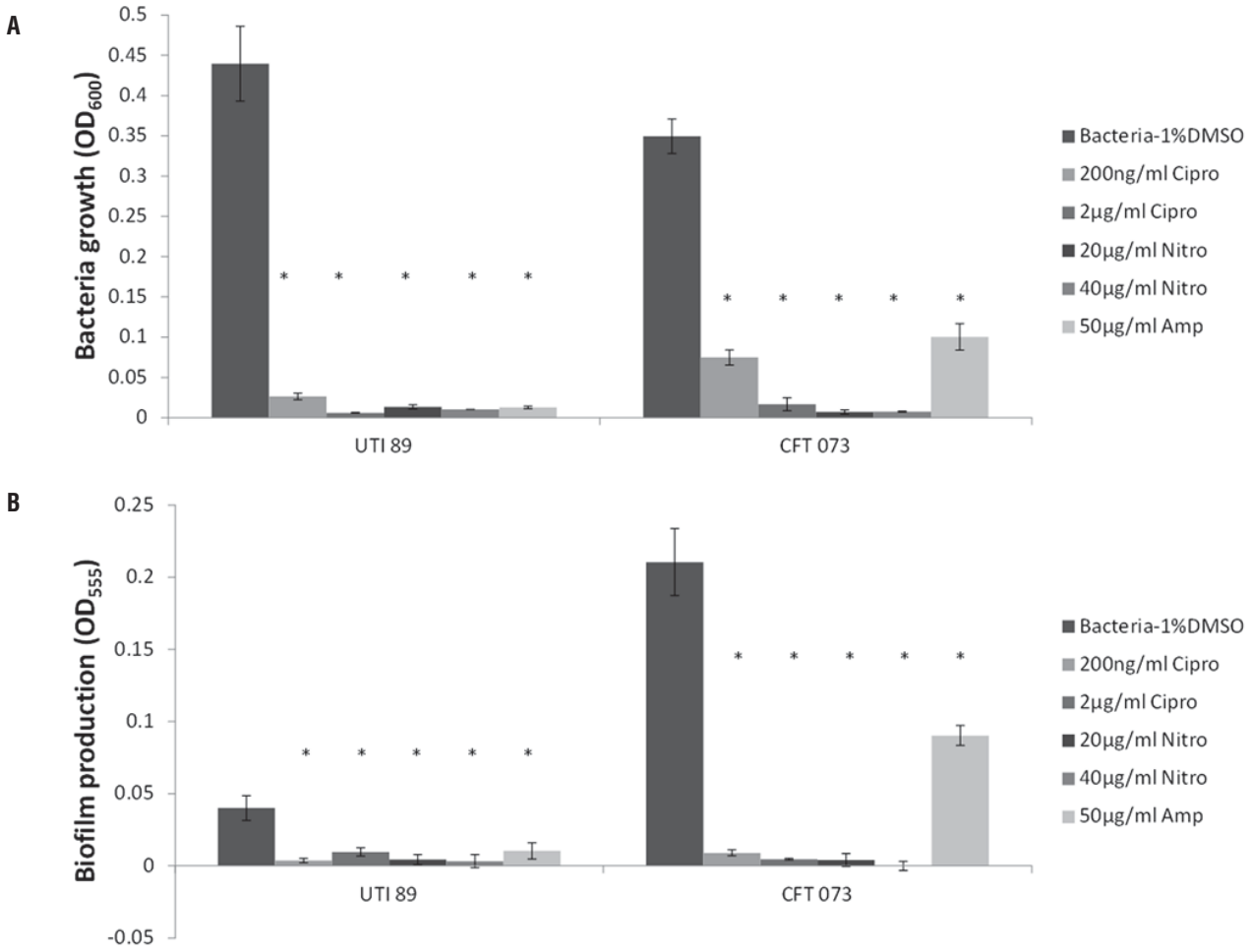
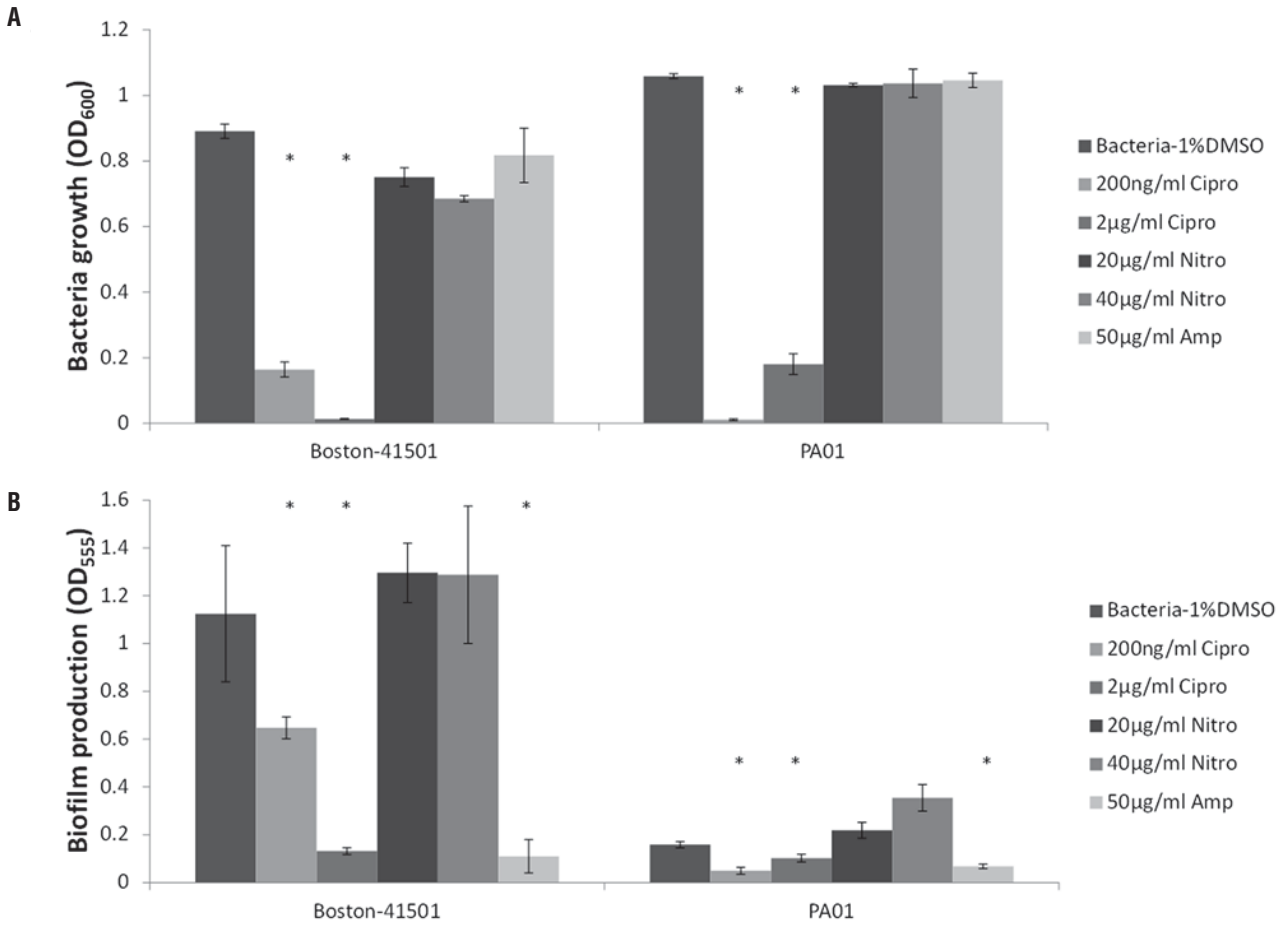


Table 1 - Inhibition of bacterial growth and biofilm formation of E. coli and P. aeruginosa by treatment of different antibiotics at initial bacterial plating.

		Antimicrobial agents[a]		
Determinations	Bacterial strains	Cipro	Nitro	Amp
Bacterial growth	UTI89	+	+	+
	CFT073	+	+	+
	Boston-41501	+	–	–
	PA01	+	–	–
Biofilm formation	UTI89	+	+	+
	CFT073	+	+	+
	Boston-41501	+	–	+
	PA01	+	–	+

[a] **Cipro =** Ciprofloxacin; **Nitro =** nitrofurantoin; **Amp =** ampicillin; **+ =** statistical significance; **- =** without statistical significance.

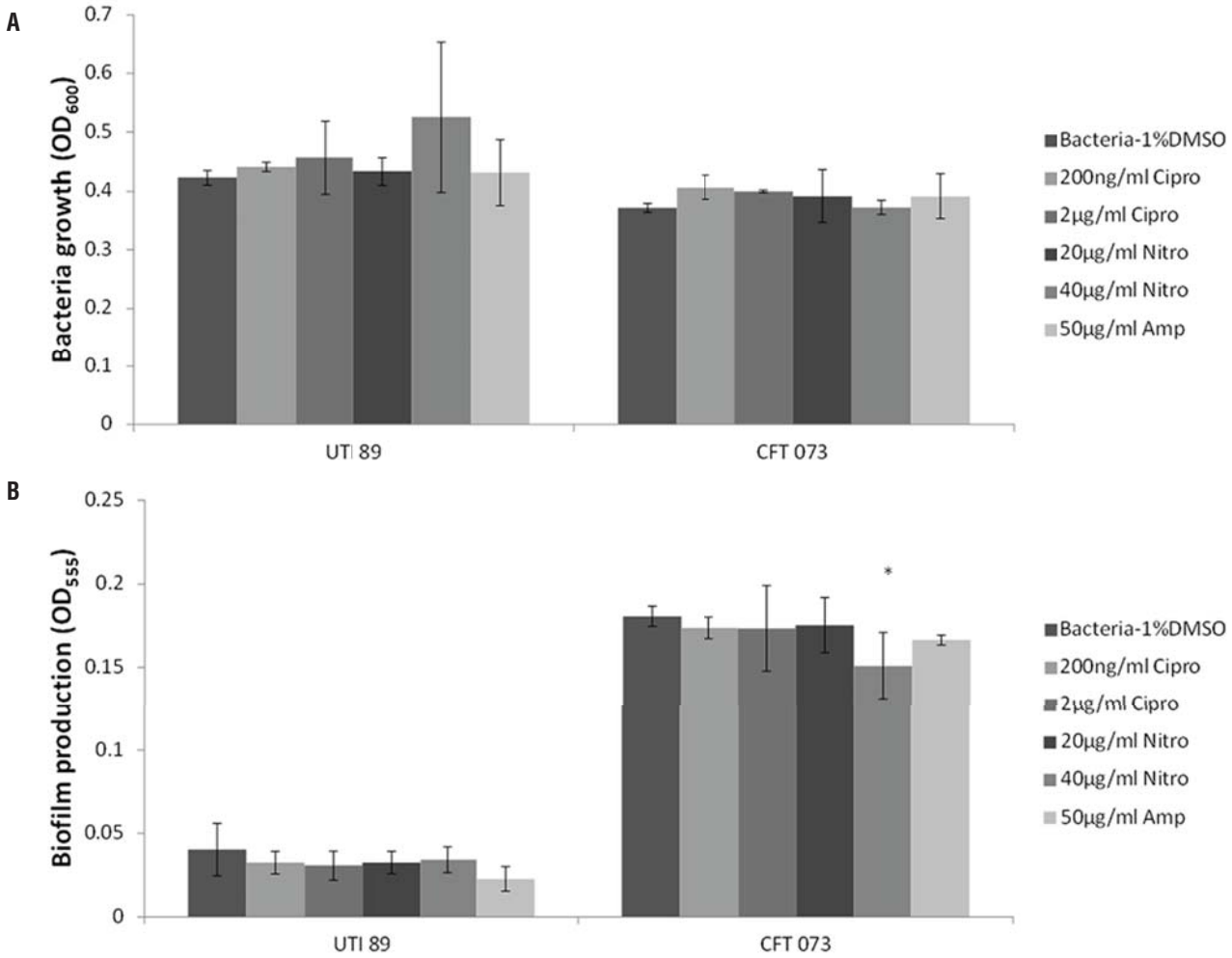
Figure 4 - Treatment of P.aeruginosa with antibiotics from Time 0. Asterisks (*) show statistical significance as compared to bacteria not treated (P<0.05).



large proportion of women after a first UTI episode (13). Different theories have been proposed to explain recurrence, including periurethral colonization and persistence of quiescent intracellular reservoirs inside a protective biofilm in the bladder (14). Other factors such as estrogen deficiency, the composition of the surface glycosaminoglycan of the bladder, the host response, and the acidity of the urine have also been implicated in this complex process. Even when patients are treated with culture-directed antibiotic therapy, some will develop a clinical lack of response, prompting a switch to a different antibiotic regimen after a few days, while others seem to respond initially but promptly recur after the completion of the antibiotic treatment course. These clinical observations suggest several plausible mechanisms, including a sub-therapeutic concentration of urinary antibiotics to effectively eradicate the bacterial infection, or an intra-vesical mechanism of bacterial protection against the antibiotics, such as a protected site for the bacteria inside a biofilm.

In this study, we selected bacteria known to be good biofilm formers, such as uropathogenic E.coli UTI89 and CFT073. We added two strains of P. aeruginosa as these bacteria are capable of producing extremely robust and generous biofilms and are among the most challenging UTIs to eradicate in patients. Since most bacteria, including those used in this study, form biofilms, we decided not to include non-biofilm forming bacterium as a control. These strains were sub-

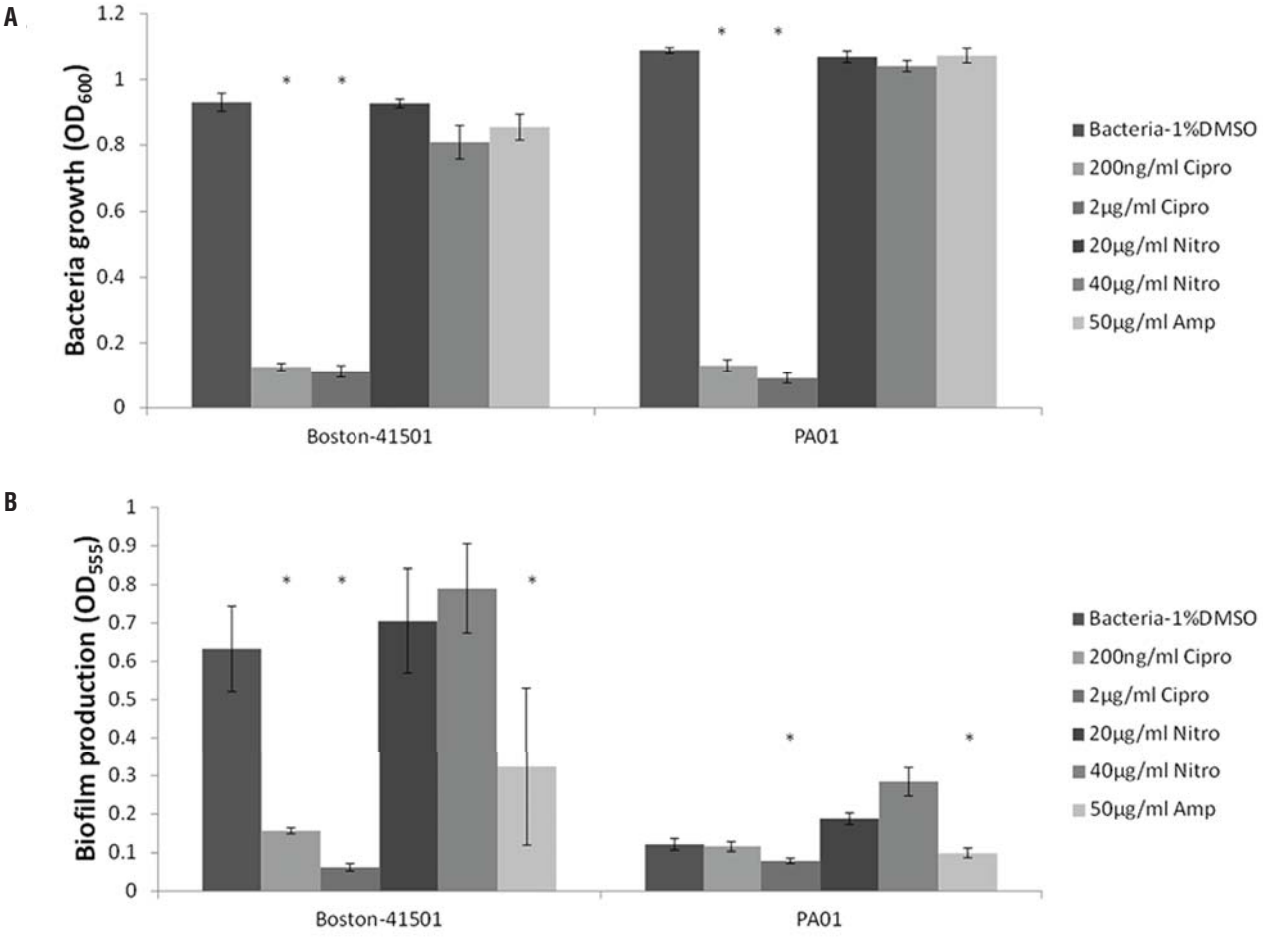
Figure 5 - Treatment of E.coli with antibiotic after untreated growth for 6 hours. Asterisks (*) show statistical significance to negative control (P<0.05).



jected to various environmental changes like movement and pH variations to determine how such changes would impact the biofilm assay results, our goal being to produce a very reliable biofilm assay for our next study. With the exception of P.aeruginosa PA01, we found that agitated conditions were more conducive to bacterial growth and biofilm formation. In general, bacterial growth of E.coli UTI89 and CFT073 reached a plateau within 6 hours after plating; however, biofilm formation continued. Although bacterial growth between CFT073 and UTI189 was similar, CFT073 produced more biofilm than UTI89. P.aeruginosa Boston-41501 reached a bacterial growth plateau at 24 hours but biofilm continued to grow in a time-dependent manner. P.aeruginosa PA01 likewise reached a growth plateau at 24 hours and biofilm production reached a plateau at 6 hours in agitated conditions. However, PA01 was able to continue producing biofilm in a time-dependent manner in static conditions (Figure-1). With our findings, we established that agitated conditions would be optimal for our future work.

Since urine pH in patients varies from pH 5 to 8, we investigated whether variations in pH would impact the bacterial growth or biofilm formation of each strain. Our results indicated that, except in more acidic conditions (pH 5), variations

Figure 6 - Treatment of P.aeruginosa with antibiotics after untreated growth for 6 hours. Asterisks (*) show statistical significance when compared to negative control (P<0.05).



in pH did not play a significant role in the inhibition of bacterial growth or biofilm production. For P.aeruginosa, at 48 hours, the strain of bacteria was able to overcome pH variations and neutralize the acidic conditions (Figure-2).

Equipped with a reliable biofilm formation assay, we then tested the effect of some of the most widely prescribed antibiotics for UTI to elucidate bacterial growth and biofilm production patterns at different time points and with different antibiotic concentrations (8, 15). Immediate treatment of either E.coli strains with Ciprofloxacin, Nitrofurantoin, or Ampicilin at various concentrations showed significant decreases in both bacterial growth and biofilm production (Figure-3 and Table-1). However, we observed a different behavior with both P.aeruginosa strains when treated immediately with antibiotics: only Ciprofloxacin was able to significantly inhibit both bacterial growth and biofilm production at both concentrations for both strains (Figure-4). A key finding of this study was that when administered at 6 hours, none of these antibiotics were able to stop E.coli bacterial growth or biofilm formation. Furthermore, Nitro increased biofilm production for P.aeruginosa, seemingly serving as a nutrient factor.

Clinically, it would be difficult to treat patients with UTI symptoms with antibiotics immediately, and even so, the biofilm may be completely formed when symptoms start. This observation could explain the trend to UTI recurrence in some women (16). In fact, some patients known to experience recurrent episodes of urinary tract infections are at times prescribed antibiotics to

have on hand to treat themselves as soon as their symptoms restart. This approach has several shortcomings, including the potential for unnecessary treatments in the absence of a culture-proven urinary tract infection and an increased risk of bacterial resistance over time. On the other hand, clinicians are sometime tempted to empirically initiate therapy while waiting for the culture results to return. Considering our data in selected strains of E.coli and P.aeruginosa known for their biofilm formation performance, such a clinical decision may be very relevant. However, caution is required as one limitation of this study is the complexity of the biofilm and the challenges of extending in vitro observations to the in vivo situation. Although there is mounting evidence on the role of biofilm resistance to antibiotics as part of the recurrence process (17), the data in humans remains weak.

Nitrofurantoin has recently been incriminated for its long-term risks (pulmonary fibrosis, peripheral neuropathy, dosing adjustment in renal insufficiency) (18, 19) to the point that its current use requires sometimes a complex clearance process. It is important to note its counter-effect leading to biofilm growth stimulation in this study, which may explain its lack of initial effectiveness or durable response even in patients with culture--proven sensitivity.

In summary, antibiotics can be very effective for the uropathogenic E.coli strains selected in this study if the treatment can be applied early on. For P.aeruginosa strains, Ciprofloxacin is the best agent to inhibit bacterial growth and biofilm production even when the agent is given when bacterial growth reached a plateau. In some situations, Nitrofurantoin can stimulate biofilm production.

ABBREVIATIONS

UTI = urinary tract infections
UPEC = uropathogenic *Escherichia coli*

CONFLICT OF INTEREST

None declared.

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